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Tuberculosis and Other Mycobacterial Infections

March 2017

Oil on canvas. 39.4 in by 35.4 in/100 cm × 90 cm. Kunsthalle Bremen, Der Kunstverein, Bremen, Germany

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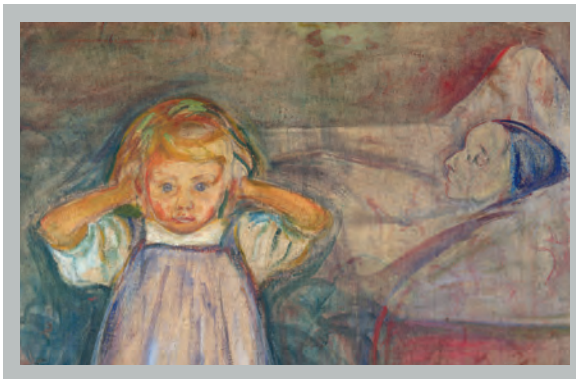

On the Cover

Edvard Munch (1863–1944) *Das Kind und der Tod*, 1899 (*The Child and Death*, 1899) (detail).

Oil on canvas. 39.4 in by 35.4 in/100 cm × 90 cm. Kunsthalle Bremen, Der Kunstverein, Bremen, Germany

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Medscape EDUCATION ACTIVITY

Epidemiology of *Mycobacterium bovis* Disease in Humans in England, Wales, and Northern Ireland, 2002–2014

Despite slightly increased cases in these areas, human infection with this cattle pathogen remains rare.

Related material available online: http://wwwnc.cdc.gov/eid/article/23/3/16-1408_article

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Three Cases of Neurologic Syndrome Caused by Donor-Derived Microsporidiosis

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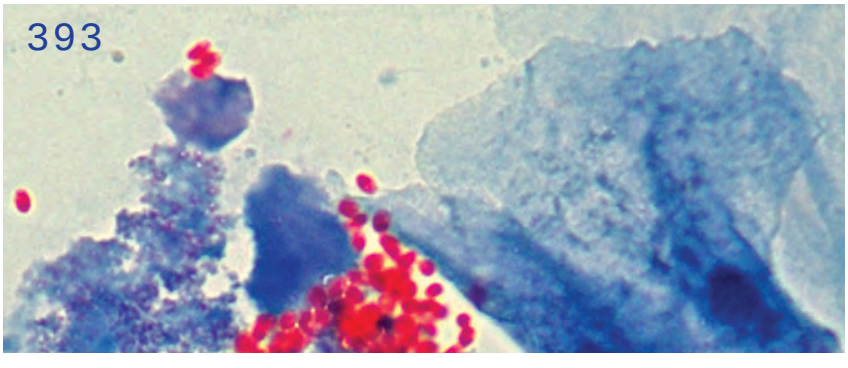

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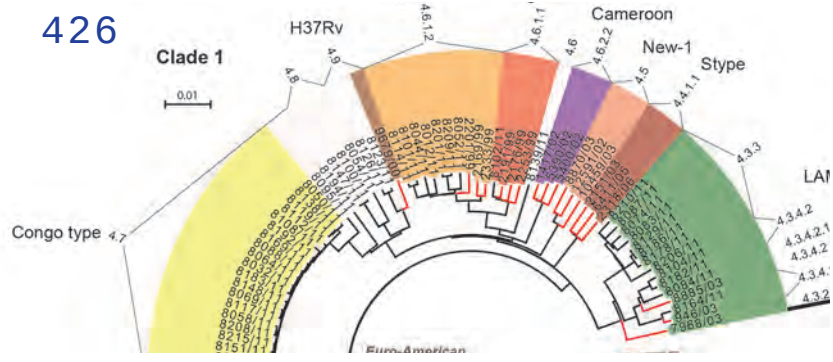

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About the Cover

Vol. 22, No. 7

The name of author Felix Drexler was misspelled in **Hepatitis E Virus Infection in Dromedaries, North and East Africa, United Arab Emirates, and Pakistan, 1983–2015.**

Vol. 23, No. 2

The key in the Figure 1 inset should have referred to hepatitis A and E in **Changing Epidemiology of Hepatitis A and Hepatitis E Viruses in China, 1990–2014.**

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http://wwwnc.cdc.gov/eid/page/spotlight-topics

Epidemiology of *Mycobacterium bovis* Disease in Humans in England, Wales, and Northern Ireland, 2002–2014

Jennifer A. Davidson, Miranda G. Loutet, Catherine O'Connor, Cathriona Kearns, Robert M.M. Smith, Maeve K. Lalor, H. Lucy Thomas, Ibrahim Abubakar, Dominik Zenner

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Release date: February 15, 2017; Expiration date: February 15, 2018

Learning Objectives

Upon completion of this activity, participants will be able to:

- Identify the demographic features of *Mycobacterium bovis* cases in England, Wales, and Northern Ireland from 2002–2014, based on a national cohort study
- Compare demographic and clinical characteristics of human tuberculosis caused by *M. bovis* and *M. tuberculosis* notified cases
- Recognize potential exposures, including genotyping comparison between human cases, that may indicate acquisition of *M. bovis* in humans.

CME Editor

P. Lynne Stockton Taylor, VMD, MS, ELS(D), Technical Writer/Editor, *Emerging Infectious Diseases. Disclosure: P. Lynne Stockton Taylor, VMD, MS, ELS(D), has disclosed no relevant financial relationships.*

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Despite control efforts, *Mycobacterium bovis* incidence among cattle remains high in parts of England, Wales, and Northern Ireland, attracting political and public health interest in potential spread from animals to humans. To determine incidence among humans and to identify associated factors, we conducted a retrospective cohort analysis of human *M. bovis* cases in England, Wales, and Northern Ireland during 2002–2014. We identified

357 cases and observed increased annual case numbers (from 17 to 35) and rates. Most patients were ≥65 years of age and born in the United Kingdom. The median age of UK-born patients decreased over time. For 74% of patients, exposure to risk factors accounting for *M. bovis* acquisition, most frequently consumption of unpasteurized milk, was known. Despite the small increase in case numbers and reduction in patient age, *M. bovis* infection of humans in England, Wales, and Northern Ireland remains rare.

After the 1960s, the number of human cases of tuberculosis (TB) caused by *Mycobacterium bovis* decreased significantly in England, Wales, and Northern Ireland, coinciding with widespread implementation of milk product pasteurization and national bovine TB control programs (*1*–*3*). During the past 2 decades in these 3 countries, an average of 30 cases of *M. bovis* in humans occurred annually; numbers decreased in the early 2000s before again increasing (*4*–*6*). During the same period, incidence of *M. bovis* in cattle herds in parts of England, Wales, and Northern Ireland increased substantially but has now plateaued (*4*,*7*–*9*).

M. bovis control (*2*,*7*,*10*,*11*) attracts political, public health, and media interest because of potential spread from animals to humans, effects on animal health and trade (*1*), and the role of wildlife in the transmission cycle (*12*). Highly visible interventions, including wildlife management to prevent transmission to livestock, are used to attempt to control *M. bovis* spread (*6*,*9*,*10*), thereby protecting human health.

Compared with other countries in western Europe, the rate of TB among humans in the United Kingdom is high: 9.6 cases/100,000 population (6,240 cases) in 2015 (*13*). Most TB cases occurred in those born abroad, who probably acquired infection before entering the United Kingdom. Although only 1.1% (42 cases) of culture-confirmed TB cases were caused by *M. bovis* (*6*), it remains a public health priority.

The drivers of the epidemiology of *M. tuberculosis* are well described (*13*–*15*). However, there is comparatively less information on the sources of *M. bovis* in humans, other than the recognized risks of unpasteurized milk consumption and close contact with infected cattle (*1*,*3*). We provide an update on the demographic characteristics of humans with *M. bovis* disease in England, Wales, and Northern Ireland (*16*). To address the gap in knowledge regarding lesser known sources of acquisition, we describe the demographic and clinical characteristics of humans with TB caused by *M. bovis* compared with *M. tuberculosis*. In addition, we describe potential human exposures that may indicate *M. bovis* acquisition and include a genotyping comparison of the causative organisms.

Materials and Methods

Study Population and Definitions

Our retrospective cohort study included all human *M. bovis* patients in the descriptive analysis. To describe demographic and clinical characteristics associated with *M. bovis* disease, we compared all *M. bovis* notified patients with all *M. tuberculosis* notified patients. Potential exposures to risk factors associated with *M. bovis* acquisition were collected through a questionnaire and limited to *M. bovis* cases identified during 2006–2014, when the questionnaire return rate was high (>80%).

An *M. bovis* case was defined as a culture-confirmed human case of TB speciated as *M. bovis* isolated during 2002–2014. A notified *M. bovis* case was an *M. bovis* case clinically notified to the Enhanced TB Surveillance system (ETS); a nonnotified *M. bovis* case was an *M. bovis* not reported clinically to ETS. An *M. tuberculosis* notified case was defined as a culture-confirmed human case of TB speciated as *M. tuberculosis* isolated during 2002–2014 and clinically notified to ETS.

Data Collection

Results from culture-positive laboratory isolates were sent from *Mycobacterium* reference laboratories in England, Wales, and Northern Ireland to Public Health England. These results were matched with notified TB cases from ETS, used for statutory notification of TB, by use of a probabilistic matching method (*17*).

Data on demographics (age, sex, ethnicity, country of birth, time since UK entry, address, and occupation); clinical factors (site of disease and previous diagnosis); and social risk factors (current or past imprisonment, homelessness, drug and alcohol misuse) were obtained from ETS notifications. For nonnotified *M. bovis* cases, the only patient demographic information available was age, sex, and address; the disease site was inferred from specimen site. For analysis, we used the age groups 0–14, 15–44, 45–64, and ≥65 years and the ethnic groups white, black African, Indian subcontinent (Indian, Pakistani, and Bangladeshi grouped together), and other. After assignment to a geographic area of residence based on address, the place of residence was classified as rural or urban by using 2011 census classifications (*18*).

After identification of an *M. bovis* case (based on phenotypic, PCR, and genotypic methods [*19*,*20*]), a questionnaire (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/23/3/16-1408-Techapp1.pdf) (*21*) was issued to collect information on potential recognized current or past *M. bovis* exposures. These exposures were contact with a human TB patient, travel (for ≥2 weeks) to or residence in a country with high TB incidence (defined as having an estimated rate of ≥40 cases/100,000 population during 2002–2014), consumption of unpasteurized milk product,

occupational contact with animals, physical contact with wild (nondomestic) animals, and physical contact with any animal with TB (including pets).

M. bovis Trend Analysis

We calculated incidence rates per 100,000 population by using mid-year population estimates produced by the UK Office for National Statistics (*22*). We used Poisson regression to calculate the incidence rate ratio to assess the trend in *M. bovis* incidence over time. We used a nonparametric test for trend across ordered groups to assess the age trend of *M. bovis* patients and the χ^2 test for trend to assess the proportion of *M. bovis* among culture-confirmed TB cases.

Factors Associated with *M. bovis* Disease and *M. tuberculosis* Disease

Demographic and clinical characteristics for *M. bovis* notified patients were compared with those of *M. tuberculosis* notified patients by using univariable and multivariable logistic regression to calculate odds ratios to identify factors associated with *M. bovis* disease. A forward stepwise multivariable logistic regression model was used, including sex and all variables with a p value <0.2 in univariable analysis; likelihood ratios were assessed after each stepwise addition to the model. In addition, we conducted a stratified analysis based on place of birth (UK-born/non–UK-born). A p value of <0.05 was considered statistically significant. We tested interactions between biologically and statistically plausible variables in the model by using likelihood ratios. All analyses were conducted by using Stata 13.1 (StataCorp LLC, College Station, TX, USA).

Exposures to Risk Factors Associated with *M. bovis* Disease

To identify frequent exposure to risk factors among the cohort, we used case exposure history, as collected through the questionnaire (online Technical Appendix), for descriptive analysis. In addition to obtaining questionnaire information about contact with another human TB patient, for culture-positive isolates identified during 2010–2014, we also obtained 24-loci mycobacterial interspersed repetitive unit–variable tandem repeat (MIRU-VNTR) strain typing results (*20*) from *Mycobacterium* reference laboratories. This information enabled us to identify strain type clusters, defined as ≥2 human TB cases with indistinguishable MIRU-VNTR profiles (or with an indistinguishable profiles but with 1 case only typed to 23 loci), Clustered cases were further investigated to identify possible epidemiologic links, the identification of which suggest recent human-to-human transmission (*23*).

Results

Demographics of *M. bovis* Patients

For 2002–2014, we identified 357 culture-confirmed cases of *M. bovis* disease in humans. During this time, the proportion of all culture-confirmed TB cases speciated as *M. bovis* increased from 0.4% to 0.9% (p<0.001). Annual case numbers ranged from 17 in 2002 to 35 in 2014, and the incidence rate fluctuated between 0.03 and 0.06 cases/100,000 population (Figure 1); the incidence rate ratio per year was 1.04 (95% CI 1.01–1.07). Overall, 92.2% (329/357) of *M. bovis* cases were notified to ETS; since 2011, all identified cases have been notified.

Among 297 *M. bovis* patients for whom place of birth was recorded, 214 (72.1%) were born in the United Kingdom. The most frequent countries of birth for the others were Nigeria (18 patients), Morocco (9 patients), and India (8 patients). The age distribution differed significantly between those born and not born in the United Kingdom (p<0.001) (Table 1). The median age of UK-born patients fluctuated over time, from 71 years (interquartile range 60–76)

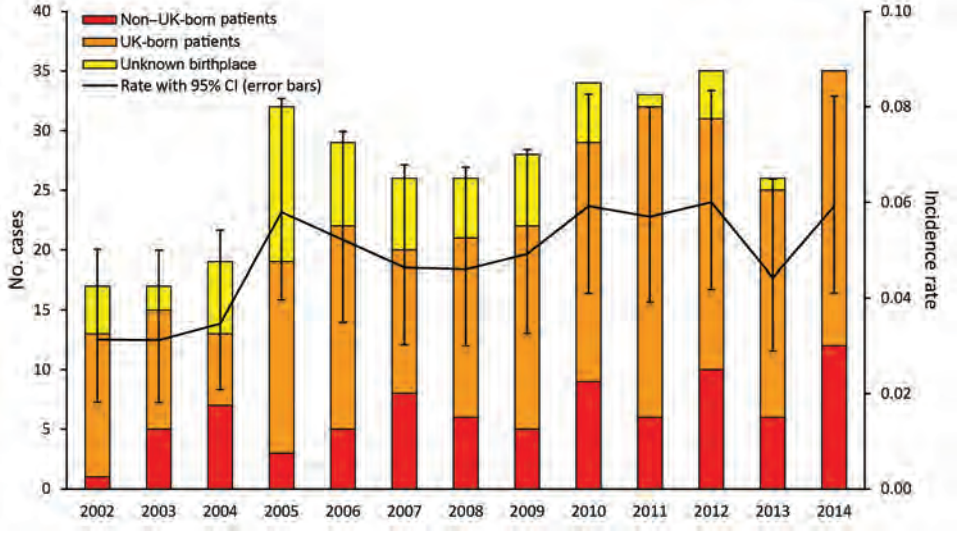

Figure 1. Annual number and incidence rate (no. cases/100,000 population) of notified *Mycobacterium bovis* cases by patient place of birth, England, Wales, and Northern Ireland, 2002–2014. Unknown place of birth includes notifications with an unknown place of birth and cases that have not been notified.

in 2002 to 53 years (interquartile range 35–79) in 2014 (p = 0.099), as did the proportion of cases by age group (Figure 2). Only 6 *M. bovis* cases in patients <15 years of age were reported (all 11–14 years of age).

For all 3 countries, the highest proportion of *M. bovis* patients resided in London, England (18.5%; 66/357), followed by the South West (15.4%; 55) and West Midlands regions of England (13.2%; 47) (Figure 3, panel A). However, the incidence rate was highest in Northern Ireland (0.11 cases/100,000 population), followed by the South West (0.08/100,000) and West Midlands (0.07/100,000) regions. The highest proportion (71.9%; 41/57) of *M. bovis* patients not born in the United Kingdom lived in London. In comparison, 85.1% (40/47) and 82.1% (32/39) of patients from the South West and West Midlands, respectively, were born in the United Kingdom.

Comparison between Notified *M. bovis* and *M. tuberculosis* Patients

Univariable analysis showed that, when compared with *M. tuberculosis* notified patients, *M. bovis* notified patients were more likely to be ≥45 years of age, born in the United Kingdom, of an ethnic group other than that of the Indian subcontinent, live in a rural area, and work in agricultural or animal-related occupations. *M. bovis* patients were less likely than *M. tuberculosis* patients to have pulmonary disease. Multivariable analysis showed that the same factors, other than age, were independently associated with *M. bovis*; only those ≥65 years of age were more likely to have *M. bovis* disease. The strongest risk factor for *M. bovis* disease was working in an agricultural or animal-related occupation (adjusted odds ratio 29.5, 95% CI 16.9–51.6; Table 2). The model showed no interactions between explanatory variables. Analysis stratifying by place of birth (UK-born vs. non–UK-born) indicated that the same variables were significant.

M. bovis Patient Exposure to Risk Factors

Of the 272 *M. bovis* patients identified during 2006–2014, exposure questionnaires were completed for 241 (88.6%). Of these, 179 (74.3%) reported exposure to at least 1 risk factor for *M. bovis* acquisition; 78 (43.6%) reported 1 exposure, 57 (31.8%) 2 exposures, 28 (15.6%) 3 exposures, and 16 (8.9%) 4 exposures. For 6 patients, no exposure was known; for the remaining 56 patients, data were missing for ≥1 risk factor and the patients could not be classified as not having been exposed to a risk factor.

The most frequently reported exposure was consumption of unpasteurized milk products (65.7%, 109/166; Table 3); proportions reporting this factor were

Table 1. Characteristics of patients with *Mycobacterium bovis* disease, England, Wales, and Northern Ireland, 2002–2014*

Characteristic†	All patients, no. (%), n = 357‡	UK-born patients, no. (%), n = 214§	Non–UK-born patients, no. (%), n = 83¶
Age group, y			
0–14	6 (1.7)	4 (1.9)	2 (2.4)
15–44	106 (29.7)	39 (18.2)	54 (65.1)
45–64	70 (19.6)	45 (21.0)	12 (14.5)
≥65	175 (49.0)	126 (58.9)	15 (18.1)
Male sex	196 (55.1)	130 (60.8)	37 (44.6)
Ethnicity			
White	230 (73.0)	199 (93.9)	15 (18.5)
Black African	37 (11.8)	2 (0.9)	35 (43.2)
Indian subcontinent	16 (5.1)	3 (1.4)	9 (11.1)
Other	32 (10.2)	8 (3.8)	22 (27.2)
Time since entered United Kingdom, y			
<2	NA	NA	10 (14.7)
2–5	NA	NA	17 (25.0)
6–10	NA	NA	20 (29.4)
>10	NA	NA	21 (30.9)
Place of residence			
Rural	86 (24.9)	62 (29.0)	9 (10.8)
Urban	259 (75.1)	152 (71.0)	74 (89.2)
Pulmonary TB#			
Yes	199 (56.9)	131 (61.5)	38 (45.8)
No	151 (42.3)	82 (38.5)	45 (54.2)
≥1 social risk factor**	12 (7.9)	6 (5.9)	5 (11.1)
Previous TB diagnosis	17 (6.1)	13 (6.7)	5 (5.4)

*IQR, interquartile range; NA, not applicable; TB, tuberculosis.
†Sex, age, and site of disease reported for all cases (excluding breakdowns by birth in or not in the United Kingdom); all other characteristics reported only for notified cases.
‡Median age (IQR) 58 (36–77) y.
§Median age (IQR) 70 (52–79) y.
¶Median age (IQR) 35 (28–58) y.
#Pulmonary TB with or without extrapulmonary TB, those recorded as "no" had exclusively extrapulmonary TB.
**Data only available from 2010 on.

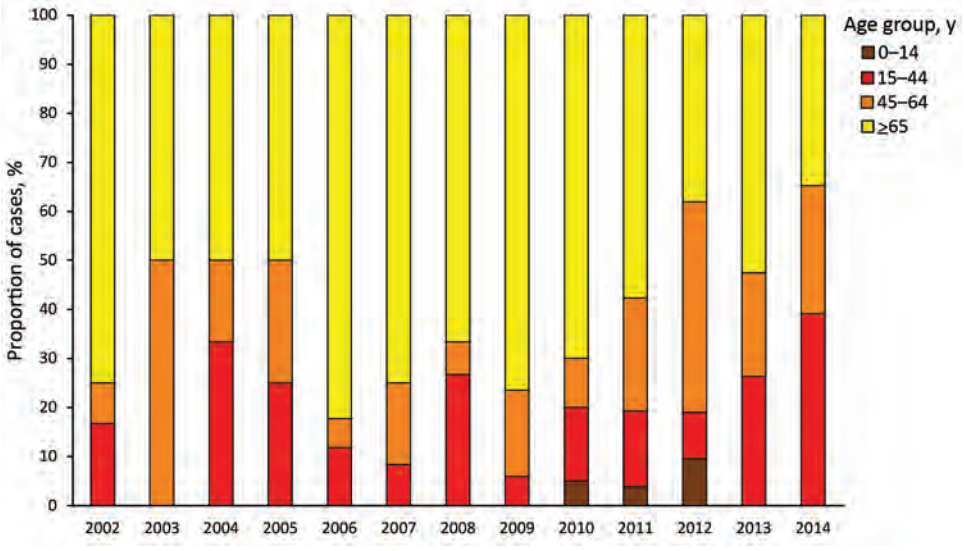

Figure 2. Annual number of notified UK-born *Mycobacterium bovis* cases, by patient age group, England, Wales, and Northern Ireland, 2002–2014.

similar among those born in the United Kingdom and those born elsewhere. Among those for whom the most recent consumption of unpasteurized milk product was known, most (85.9%, 55/64) had consumed the product >5 years before TB diagnosis; 42.2% (27/64) were >50 years of age before diagnosis. No change in the age distribution of patients consuming unpasteurized milk was identified over time; most (56.0%, 61/109) were ≥65 years of age.

Contact with a human TB patient was reported by 18.2% (33/181), but for most, recorded information was insufficient to identify the contact, particularly if the contact was not recent. Where known, 80.8% (21/26) of contacts occurred >5 years before TB diagnosis. From 24-loci MIRU-VNTR strain typing data available during 2010–2014, a total of 48.7% (57/117) of patients (of which 46 were born in the United Kingdom and 9 were not) were in 15 *M. bovis* strain type clusters. One cluster contained exclusively patients not born in the United Kingdom and 7 exclusively born in the United Kingdom; 2 of the latter clusters contained the only epidemiologically linked human patients, each with a pair of household contacts.

Recent acquisition of infection cannot be directly measured, but the rate of *M. bovis* disease among children, along with their exposures, can provide an indirect indicator of recent acquisition. Exposure information was available for 5/6 *M. bovis* patients <15 years of age and suggested potential overseas acquisition; 5 had traveled to a country where TB incidence was high, 1 of whom had consumed unpasteurized milk while abroad. For 1 child not born in the United Kingdom, a questionnaire response was not obtained.

Overall, among those for whom location of exposure was known, 59.1% (97/164) of patients were exposed to ≥1 risk factor in the United Kingdom (Table 3). Among those not born in the United Kingdom, 18.0% (9/50) were known to have been exposed to a risk factor while in the United Kingdom, but 4 of the 9 also were exposed outside the United Kingdom.

Discussion

Our findings confirm that *M. bovis* disease remains rare among humans in England, Wales, and Northern Ireland. Over the study period, the annual rate of *M. bovis* disease and the proportion of culture-confirmed TB cases with *M. bovis* identified as the cause displayed a small but statistically significant increase; annual case numbers for the past 10 years were similar to those for the early 1990s (*4*). Although speciation has improved from the use of strain typing results (*19*,*20*), this improvement is unlikely to account for all of the increase identified. Although the previous study by Jalava et al. (*4*) and our study overlap by 2 years, our results benefit from improved matching (*17*) between case notification and culture results from 2002 on, thereby providing improved accuracy for reporting annual case numbers.

We identified, unlike previous studies (*4*,*5*), that although the number of *M. bovis* patients not born in the United Kingdom remained low and fluctuated over time, the annual number of cases in this group increased slightly over time. Our finding may be confounded by better recording of place of birth but is not unexpected given the increase during this period in the overall number of TB patients not born in the United Kingdom (*13*). Similar to previous findings (*5*), our findings indicate that most *M. bovis* patients not born in the United Kingdom lived in urban areas, specifically London. These patients originated mostly from low-income countries where TB incidence is high and therefore are at higher risk for human-to-human transmission and animal-to-human transmission because of limited

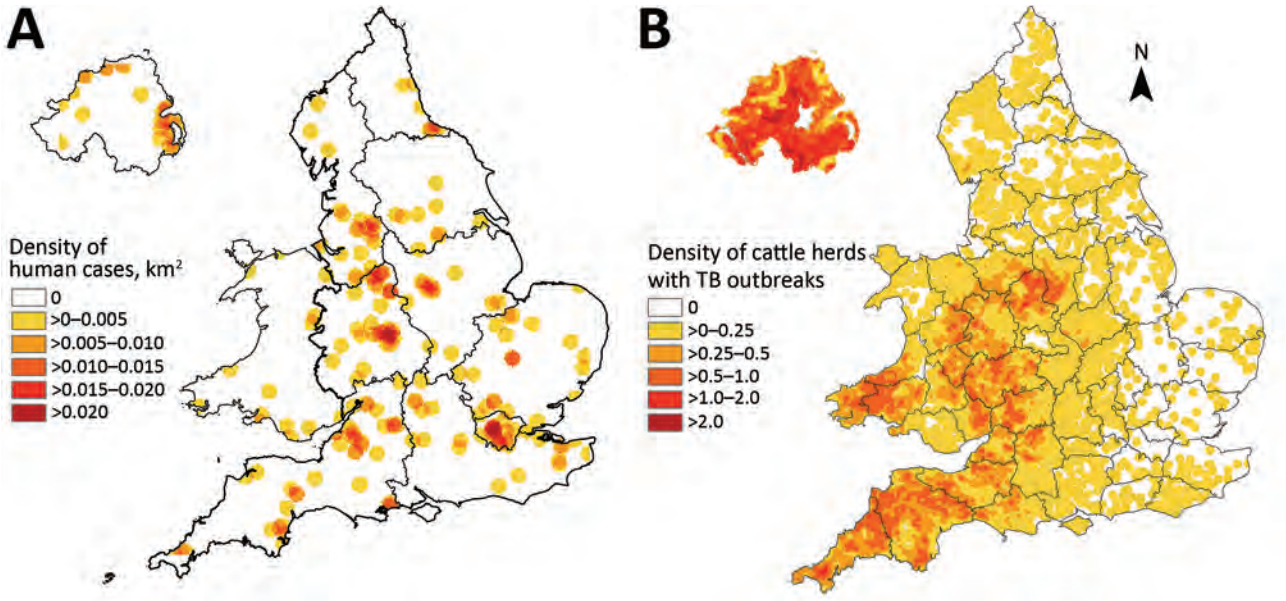

Figure 3. Cases of *Mycobacterium bovis* disease in England, Wales, and Northern Ireland, 2002–2014. A) Density of human cases. B) Density of cattle herds with TB outbreaks. This material is based on Crown Copyright and is reproduced with the permission of Land & Property Services under delegated authority from the Controller of Her Majesty's Stationery Office.

detection of *M. bovis* in animals and less frequent milk pasteurization (*24*). Thus, infection was probably acquired before arrival in the United Kingdom and less likely to be related to exposure to risk factors while in the United Kingdom. Unfortunately, speciation is not routinely conducted in many high TB burden, low-income countries (*16*), so it is difficult to identify in which countries incidence of *M. bovis* is high and whether the trends in country of birth for *M. bovis* patients not born in the United Kingdom reflect the global incidence of the disease (*24*,*25*).

We identified a decrease over time in the proportion of UK-born patients ≥65 years of age and a decrease in the median age, although neither was statistically significant. Previously, most cases in UK-born patients were the result of reactivation of infection acquired before the large rollout of pasteurization (by the 1960s) (*4*), when *M. bovis* incidence was higher. Given the length of time that widespread pasteurization has been in place, progressively fewer cases among the older population are expected as the cohort exposed before pasteurization decreases. It was unexpected that, despite this decrease, the number of *M. bovis* cases occurring in the UK-born population did not reduce over the study period. Instead, the number and proportion of younger UK-born patients increased slightly. Numbers remain small, and it is not possible to yet detect any change in exposures; however, in recent years, the media have reported increased public demand for unpasteurized milk, which, if contaminated, could result in more human infections.

No data are available to quantify unpasteurized milk production or consumption within England, Wales, and Northern Ireland. However, results from a 2012 survey of adult consumer attitudes about unpasteurized milk (*26*) showed that 33% of respondents had consumed unpasteurized milk but only 3% currently consumed unpasteurized milk. Although the proportion who had ever consumed unpasteurized milk was highest among older age groups (18–24 years, 31%; 25–44 years, 28%; 45–64 years, 38%; ≥65 years, 40%), the proportion of current consumers was higher among younger age groups (18–24 years, 7%; 25–44 years, 4%; 45–64, 1%; ≥65, 1%). It is possible that increased consumption of unpasteurized milk, as reported by the media, is contributing to the small increase in *M. bovis* cases and may contribute to a change in demographics of patients over time. Although we do not have evidence to confirm, this hypothesis could be explored further through a formal observation study. The time between unpasteurized milk consumption and onset of TB disease among the *M. bovis* patients in our cohort emphasizes that the effects of current unpasteurized milk consumption may not be observed for many years.

The results of combining routine 24-loci MIRU-VNTR typing of *M. bovis* from humans with epidemiologic data provide evidence of only occasional human-to-human *M. bovis* transmission; despite extensive follow-up of the 57 clustered cases, only 2 instances of 2 cases being epidemiologically linked were found. Only 1 prior occurrence of MIRU-VNTR–confirmed (using 15-loci typing) human-to-human transmission of *M. bovis* in the United Kingdom has been documented (*5*,*27*); it occurred before the rollout of routine prospective 24-loci MIRU-VNTR

Table 2. Demographics and risk factors for patients with *Mycobacterium tuberculosis* and *M. bovis* disease, England, Wales, and Northern Ireland, 2002–2014*

Characteristic	*M. bovis* patients, no. (%), n = 329	*M. tuberculosis* patients, no. (%), n = 58,540	Univariable analysis OR (95% CI)	Univariable analysis p value	Multivariable analysis OR (95% CI)	Multivariable analysis p value
Age group, y						
0–14	6 (1.8)	1,200 (2.1)	1.9 (0.8–4.3)	<0.001	1.5 (0.6–3.6)	<0.001
15–44	102 (31.0)	38,558 (65.9)	Referent		Referent	
45–64	61 (18.5)	10,953 (18.7)	2.1 (1.5–2.9)		1.3 (0.9–2.0)	
≥65	160 (48.6)	7,826 (13.4)	7.7 (6.0–9.9)		3.6 (2.6–5.2)	
Sex						
M	179 (54.4)	33,715 (57.7)	0.9 (0.7–1.1)	0.229	0.9 (0.7–1.1)	0.287
F	150 (45.6)	24,721 (42.3)	Referent		Referent	
UK-born						
Yes	214 (72.1)	13,576 (24.7)	7.9 (6.1–10.1)	<0.001	2.6 (1.7–4.1)	<0.001
No	83 (27.9)	71,361 (75.3)	Referent		Referent	
Ethnicity						
White	230 (73)	11,968 (21.1)	28.0 (16.8–46.4)	<0.001	14.6 (7.2–26.9)	<0.001
Black African	37 (11.8)	12,501 (22.1)	4.3 (2.4–7.7)		7.4 (3.7–14.9)	
Indian subcontinent†	16 (5.1)	23,285 (41.1)	Referent		Referent	
Other	32 (10.2)	8,905 (15.7)	5.2 (2.9–9.5)		7.3 (3.6–14.8)	
Occupation						
Agricultural/animal contact work	20 (7.9)	116 (0.3)	32.4 (19.8–53.0)	<0.001	29.5 (16.9–51.6)	<0.001
Other	232 (92.1)	43,698 (99.7)	Referent		Referent	
Site of disease						
Pulmonary	193 (58.8)	37,580 (64.2)	0.8 (0.6–1.0)	0.048	0.4 (0.3–0.5)	<0.001
Extrapulmonary only	135 (41.2)	20,938 (35.8)	Referent		Referent	
Place of residence						
Rural	81 (24.6)	1,944 (3.3)	9.5 (7.3–12.2)	<0.001	2.8 (2.0–3.9)	<0.001
Urban	248 (75.4)	56,380 (96.7)	Referent		Referent	

*Interactions between 1) place of birth (UK-born/non–UK-born) and all of the other variables (age, sex, ethnicity, occupation, site of disease, place of residence [rural/urban]) and 2) age and site of disease or place of residence (rural/urban) were tested. No significant interactions existed in the model.
OR, odds ratio.
†Indian, Bangladeshi, and Pakistani ethnic groups.

typing. There are also few examples of human-to-human *M. bovis* transmission in countries other than those included in this study (*28*,*29*), suggesting that such transmission is rarely identified. Overall, the proportion of clustering among *M. bovis* cases (49%) was slightly lower than that of the overall proportion among all TB cases (56%) observed in England (*13*).

This analysis also presents findings consistent with those previously reported (*4*). Although the proportion of cases among the older UK-born population seems to be decreasing, over the study period this group accounted for most cases. Our comparative analysis confirmed that the demographic profile of *M. bovis* patients differs from that of *M. tuberculosis* patients. The consumption of unpasteurized milk remained the most frequently reported exposure, and *M. bovis* patients were more likely than *M. tuberculosis* patients to work or have worked in agricultural and animal-related occupations. These findings are reassuring and show that *M. bovis* disease is still largely limited to those with recognized risk factors for infection. Few incidents involving animal-to-human transmission on farms (*1*,*30*,*31*) and a single incident of *M. bovis* transmission from a pet to its owners *32*,*33*) have occurred during the study period. Most animal-to-human transmission remains sporadic, and implementation of additional specific interventions beyond those currently in place (*1*,*2*) would be difficult.

A high proportion of UK-born patients lived in rural areas, especially across the South West and Midlands of England, where *M. bovis* incidence among cattle is high (Figure 3, panel B). Most of these patients reported consumption of unpasteurized milk or contact with animals. However, human patients without such exposures and who reside in these areas where *M. bovis* cattle incidence is high should continue to be monitored and thoroughly investigated to ensure that lesser known exposures are not missed.

Similar to our study, a study in the Netherlands identified that the highest proportion of *M. bovis* cases occurred in the older native population (50%), followed by the foreign-born population (40%) (*34*). In comparison with our study, studies from the United States found that being foreign born (in particular, being of Hispanic ethnicity) and younger were independently associated with *M. bovis* when compared with *M. tuberculosis* (*35*,*36*). The difference in demographic characteristics of *M. bovis* patients in the United States and in England, Wales, and Northern Ireland may be explained by the fact that *M. bovis* in cattle or wildlife is not frequently reported in the United States (*37*,*38*) but is more common in neighboring Mexico (*39*,*40*). Thus, the epidemiology of human *M. bovis* in England, Wales, and Northern Ireland continues to be driven by the past and, to some extent, present prevalence of disease in cattle. Given advances in molecular techniques,

Table 3. Risk factor exposures reported by patients with *Mycobacterium bovis* disease, England, Wales, and Northern Ireland, 2006–2014*

Exposure	No. characteristics/no. with information recorded (%)		
	All patients	UK-born patients	Non–UK-born patients
Consumption of unpasteurized milk products	109/166 (65.7)	74/112 (66.1)	25/37 (67.6)
In United Kingdom	66/88 (75.0)	60/64 (93.8)	1/15 (6.7)
Travel or residence in a high incidence country	77/203 (37.9)	29/126 (23.0)	44/56 (78.6)
Work related animal exposure	51/103 (49.5)	35/71 (49.3)	7/22 (31.8)
In United Kingdom	33/37 (89.2)	28/29 (96.6)	2/4 (50.0)
Contact with human patient with TB	33/181 (18.2)	21/120 (17.5)	10/42 (23.8)
In United Kingdom	36/38 (94.7)	19/19 (100)	6/8 (75.0)
Physical contact with wild animal	18/126 (14.3)	15/92 (16.3)	0/25 (0)
In United Kingdom	10/13 (76.9)	7/10 (70.0)	Not applicable
Physical contact with animal with TB	18/99 (18.2)	14/76 (18.4)	2/18 (11.1)
In United Kingdom	10/1 (90.9)	9/10 (90.0)	1/1 (100)
Pet with TB†	2	2	0
Farm animal with TB†	11	9	1
No exposure†	6	6	0

*TB, tuberculosis.
†Denominator not available.

improved understanding of animal-to-human transmission will require linking the genotyping results from animals with *M. bovis* infection in England, Wales, and Northern Ireland with data from humans.

Globally, zoonotic TB should be tackled, and the needs of those affected by *M. bovis* disease, namely those in animal-related occupations and those consuming unpasteurized milk from infected animals, should be addressed. The implementation of methods to identify *M. bovis* where culture is not possible have been highlighted as essential (*16*,*41*,*42*). Although findings from England, Wales, and Northern Ireland cannot be extrapolated even to other high-income countries, much less to high TB burden, low-income countries, our study does illustrate the value of monitoring *M. bovis* disease and the data required to do so.

Our study does have some limitations. The exposure questionnaires return rate was 89%, and some responses were missing, which could lead to some error in the estimation of exposures; in addition, nonresponders were more likely to be urban dwellers. Our comparison of *M. bovis* and *M. tuberculosis* patients was limited because exposure questionnaire information was only collected for *M. bovis* patients; therefore, animal-related exposures, travel to countries with high TB incidence, and contact with human TB patients could not be included in the analysis. In addition, patients not born in the United Kingdom, most of whom belong to Indian subcontinent ethnic groups, are more likely missed in analysis because a higher proportion have exclusively extrapulmonary disease (*43*), for which culture confirmation is lower. Approximately 60% of TB cases in England, Wales, and Northern Ireland are culture confirmed; therefore, the estimated *M. bovis* incidence presented in this article is probably an underestimate. The proportion of TB cases culture confirmed over time has remained relatively stable (*44*), so underascertainment should not affect changes in the number or proportion of TB cases caused by *M. bovis*.

In conclusion, we found that *M. bovis* disease continues to account for a small number and low proportion of total TB cases in England, Wales, and Northern Ireland. The proportion of culture-confirmed TB cases caused by *M. bovis* has increased slightly, and the age of UK-born patients has decreased. The reasons are not fully understood, and trends should continue to be monitored. For most patients, exposure to risk factors for *M. bovis* acquisition (e.g., unpasteurized milk consumption, farm work, or contact with a human TB patient) were known. The current control measures in place to prevent animal-to-human spread seem to be effective; such spread occurs in a few isolated incidents and sporadic events. However, to increase understanding of *M. bovis* transmission in England, Wales, and Northern Ireland, we recommend strengthening collaboration between animal and human health, including linking genotyping results.

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Three Cases of Neurologic Syndrome Caused by Donor-Derived Microsporidiosis

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Release date: February 16, 2017; Expiration date: February 16, 2018

Learning Objectives

Upon completion of this activity, participants will be able to:

- Distinguish the clinical presentation and course of 3 solid-organ transplant recipients infected with *Encephalitozoon cuniculi* from a single infected donor, based on an investigation from the US Centers for Disease Control and Prevention
- Evaluate diagnostic testing and confirmation of *E. cuniculi* infection in 3 solid-organ transplant recipients
- Determine management and clinical implications of *E. cuniculi* infection in 3 solid-organ transplant recipients.

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In April 2014, a kidney transplant recipient in the United States experienced headache, diplopia, and confusion, followed by neurologic decline and death. An investigation to evaluate the possibility of donor-derived infection determined that 3 patients had received 4 organs (kidney, liver, heart/kidney) from the same donor. The liver recipient experienced tremor and gait instability; the heart/kidney and contralateral kidney recipients were hospitalized with encephalitis. None experienced gastrointestinal symptoms. *Encephalitozoon cuniculi* was detected by tissue PCR in the central nervous system of the deceased kidney recipient and in renal allograft tissue from both kidney recipients. Urine PCR was positive for *E. cuniculi* in the 2 surviving recipients. Donor serum was positive for *E. cuniculi* antibodies. *E. cuniculi* was transmitted to 3 recipients from 1 donor. This rare presentation of disseminated disease resulted in diagnostic delays. Clinicians should consider donor-derived microsporidial infection in organ recipients with unexplained encephalitis, even when gastrointestinal manifestations are absent.

Each year in the United States, ≈30,000 solid organ transplants are performed (*1*). It is estimated that 0.3%–2.0% of transplants may be complicated by donor-derived infection, most commonly of bacterial or viral origin (*2*–*4*). Parasitic and fungal infections, including microsporidiosis, make up a minority of donor-derived infections (*2*–*4*). Maintaining a high index of suspicion for donor-derived infection in solid organ transplant recipients and prompt investigation of illness suspected to be donor derived are critical because multiple recipients often receive solid organs from a common donor. Thus, identification of donor-derived infection in 1 recipient has consequences for the clinical care of the other recipients.

Potential donor-derived disease transmission events are reported to the Organ Procurement and Transplantation Network (https://optn.transplant.hrsa.gov/) per policy and reviewed by the Network's ad hoc Disease Transmission Advisory Committee, which categorizes each by the likelihood of disease transmission. Through representation on this advisory committee, the Centers for Disease Control and Prevention (CDC), with support from state and local health departments, leads investigations of select cases of public health importance. In April 2014, CDC was notified of a renal transplant recipient hospitalized with signs and symptoms of encephalitis (Figure 1). Postmortem testing revealed infection with microsporidia, and concern was raised for donor-derived central nervous system (CNS) infection. We conducted an investigation to 1) identify other ill recipients from the common donor, 2) determine whether the illness was donor derived, and 3) make treatment recommendations for the surviving recipients.

Methods

Medical records from the organ donor and all recipients were reviewed to describe clinical course, diagnostic testing, and event timelines. Epidemiologic investigation identified other ill recipients. The donor's next of kin were interviewed to ascertain potential risk factors for infectious diseases, including microsporidiosis. Because this investigation was considered a public health emergency, it was not subject to institutional review board approval.

A serum sample from the organ donor and available specimens from all recipients were sent to CDC for further testing to identify an infectious etiology. These tests included histopathology, in vitro culture immunohistochemistry (IHC), PCR, and transmission electron microscopy. Serum immunofluorescence antibody testing (IFA) was performed on available serum samples.

Cell Culture and Serum Testing

We inoculated recipient urine specimens onto monkey kidney cells (Vero E6) and human embryonic lung fibroblasts by using established methods (*5*). Cultures were treated with 2% sodium dodecyl sulfate and passaged to eliminate cytomegalovirus. For IFA, *Encephalitozoon cuniculi* reference strain cultures were suspended in phosphate-buffered saline (PBS) at a concentration of 10^8 spores/mL. We added 10 μL of this suspension to each well (10^6 spores/well), allowed the slides to air dry, and stored them at -80°C before use.

Serum samples were diluted 1:2 in 25 μL PBS along with previously established positive (titer 1:4,096) and negative (titer 1:32) control serum obtained from the CDC free-living amebic infections laboratory. The serum samples were then diluted to 1:4,096 by 2-fold dilution. We added 10 μL from each dilution to the wells of the previously made *E. cuniculi* IFA slides, which were then incubated at 37°C for 30 min. The slides were washed 3 times in PBS before the addition of a 1:100 dilution of fluorescein isothiocyanate–conjugated goat anti-human IgG (Cappel Laboratories, Cochranville, PA, USA) in PBS with 3 μL/mL Evans Blue (Fisher Scientific, Pittsburgh, PA, USA) counterstain. After another 30-min incubation at 37°C, the slides were again washed 3 times in PBS, dried, and mounted with glycerol mounting media. Serum titers were determined by observing the slides under a fluorescence microscope. Antibody titers were considered positive at cutoffs of >1:16 for immunocompromised and >1:64 for immunocompetent persons (*6*).

Histopathology, Immunohistochemistry, and Transmission Electron Microscopy

We processed formalin-fixed paraffin-embedded tissues by using standard histologic methods. IHC was performed by using a polymer-based indirect immunoalkaline phosphatase detection system with a fast red chromogen kit (Biocare Medical, Concord, CA, USA). The microsporidia IHC assay used rabbit anti–*E. cuniculi* serum at a 1:1,000

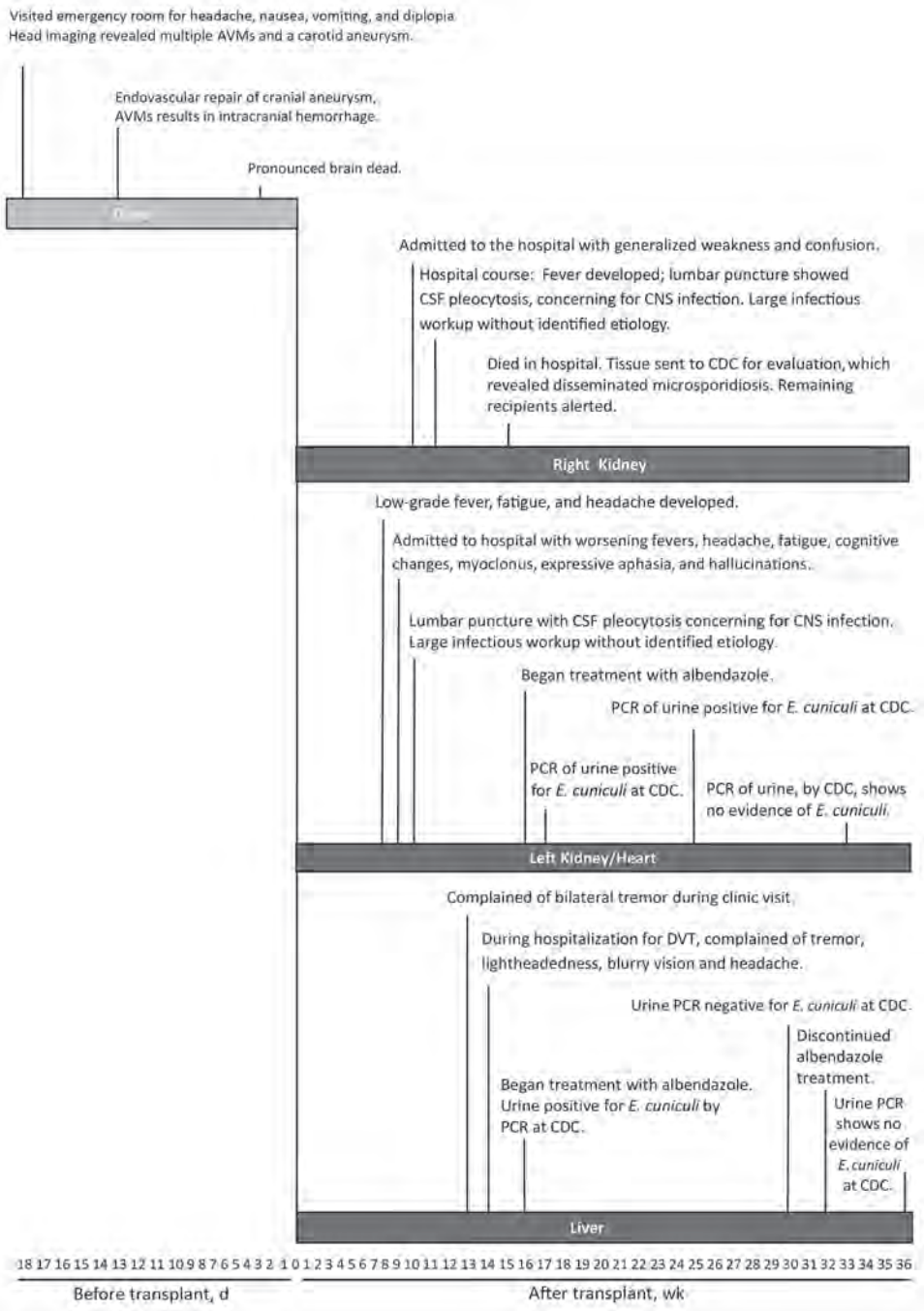

Figure 1. Timeline of events for transplant donor and 3 solid organ recipients with microsporidiosis (*Encephalitozoon cuniculi*). AVM, arteriovenous malformation; CDC, Centers for Disease Control and Prevention; CNS, central nervous system; CSF, cerebrospinal fluid; DVT, deep vein thrombosis.

dilution as previously described (*7*). In addition, IHC assays for lymphocytic choriomeningitis virus, measles virus, and *Trypanosoma cruzi* were performed as previously described (*8*–*10*).

For transmission electron microscopic examination, we evaluated a paraffin-embedded section by on-slide embedding as previously described (*11*). The urine cell pellet and microsporidia culture isolates were transferred to buffered 2.5% glutaraldehyde and 1% osmium tetroxide, embedded in a mixture of Epon substitute and Araldite (Ted Pella, Inc., Redding, CA, USA), sectioned, and stained with uranyl acetate and lead citrate.

Molecular Techniques

DNA was extracted from unpreserved clinical samples by using a DNeasy Blood & Tissue DNA extraction kit (QIAGEN, Valencia, CA, USA). DNA was extracted from the formalin-fixed paraffin-embedded tissues by using a QIAamp DNA Mini Kit (QIAGEN) as previously described (*7*,*12*). PCR primers (concentration 15 μmol/L

each) specific for *E. cuniculi* small subunit ribosomal RNA gene were used (*7*,*12*). We performed PCR by using AmpliTaq Gold PCR Master Mix (ThermoFisher Scientific, Grand Island, NY, USA) and 5 μmol of each primer at an annealing temperature of 65°C. All DNA extracts were subjected to PCR. Positive and negative controls were included in every PCR run. Any specimen that resulted in a 551-bp fragment was considered positive for the presence of *E. cuniculi* DNA.

Results

Right Kidney Recipient

The right kidney recipient was a man with history of end-stage renal disease resulting from type 2 diabetes mellitus. He received basiliximab to induce immunosuppression, and at discharge he received tacrolimus, mycophenolate mofetil, and prednisone to maintain immunosuppression. His immediate postoperative course was complicated by delayed graft function and anemia. Approximately 10 weeks after transplantation, generalized weakness and confusion developed, and he was admitted to the hospital for evaluation. Initially his clinical condition was thought to result from noninfectious causes, such as medication side effects. However, during his hospitalization, fever, pancytopenia, and acute renal failure developed, and his mental status worsened. Lumbar puncture was performed, and cerebrospinal fluid analysis revealed 10 leukocytes/μL, which led to concern for viral encephalitis. An extensive evaluation for CNS infection was initiated, and test results were negative for bacterial, fungal, viral, and parasitic causes (Table 1). Despite empirically prescribed broad-spectrum antimicrobial drugs, the recipient's illness progressed, his obtundation worsened, and hypotension required vasopressors. He died ≈15 weeks after transplantation. An autopsy was performed at the local hospital, and tissues were submitted to CDC for examination. Four days after the recipient's death, examination at CDC of the deceased recipient's renal allograft demonstrated intracellular organisms consistent with microsporidia. Clinicians caring for the other recipients were immediately notified.

Subsequent PCR of DNA extracts from the right renal allograft revealed the species to be *E. cuniculi*, and electron microscopy showed a polar tubule arrangement characteristic for *E. cuniculi* (Figure 2). The recipient's CNS tissue also was positive for *E. cuniculi* by histopathology and PCR, which showed microsporidia associated with glial nodules and the leptomeninges, the latter with perivascular inflammation. No arteritis or aneurysmal change in the vessels of the CNS were observed. Immunohistochemistry of CNS tissue also provided positive results (Table 2). IHC results were negative for lymphocytic choriomeningitis virus, measles virus, and *T. cruzi*.

Left Kidney and Heart Recipient

The recipient of the left kidney and heart was a woman in whom coronary vasculopathy and calcineurin inhibitor–induced nephropathy had developed after a cardiac transplant 21 years earlier. To induce immunosuppression before the upcoming kidney and heart transplant, she received a 5-day

Table 1. Infectious disease testing performed for 3 transplant recipients with donor-derived microsporidiosis

Pathogen	All recipients	Right kidney recipient	Left kidney/heart recipient	Liver recipient
Bacterial	Bacterial culture (blood, urine)	*Mycoplasma* *Mycobacterium tuberculosis* *Borrelia burgdorferi* *Brucella* spp. *Rickettsia* spp. *Pneumocystis jiroveci* *Legionella* spp.	*Borrelia burgdorferi* *Anaplasma/Ehrlichia* *Treponema pallidum* *Tropheryma whipplei*	*Treponema pallidum*
Viral	Cytomegalovirus Herpes simplex virus Epstein-Barr virus Parvovirus HIV	Enterovirus Lymphocytic choriomeningitis Measles virus JC virus Human herpesvirus-6 Viral fecal cultures BK virus Human T-cell lymphotropic virus Meningoencephalitis panel*	Enterovirus Adenovirus Lymphocytic choriomeningitis Measles virus JC virus Human herpesvirus-6	None
Fungal/parasitic/ other	*Cryptococcus* spp.	*Coccidioides* spp. *Aspergillus* spp. *Strongyloides* spp. *Schistosoma* spp. *Babesia* spp.	(1–3)β-D-glucan *Galactomannan* *Coccidioides* spp. *Cryptococcus* spp. *Toxoplasma gondii* *Babesia* spp. 14–3-3 testing for prion disease (cerebrospinal fluid)	*Toxoplasma gondii*

*Panel contains the following viruses: West Nile, Lacrosse, Eastern equine encephalitis, St. Louis encephalitis, Western equine encephalitis, lymphocytic choriomeningitis, herpex simplex 1 and 2, adenovirus, influenza A and B, measles, mumps, varicella, coxsackie A and B, echovirus, cytomegalovirus.

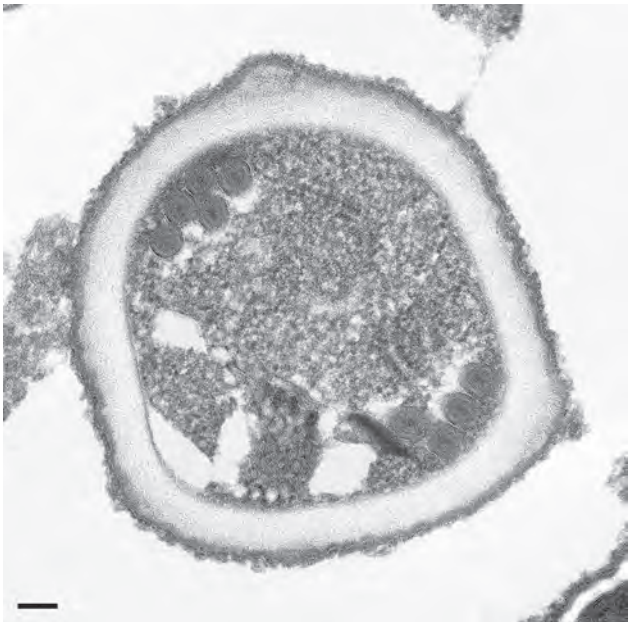

Figure 2. Transmission electron microscopy of microsporidia identified in allograft samples from right kidney recipient. The organism shows cross-sections through the polar tube with up to 6 coils and a unikaryotic nucleus, which is characteristic of *Encephalitozoon cuniculi*. Scale bar indicates 100 nm.

course of antithymocyte globulin; to maintain immunosuppression, she received tacrolimus, mycophenolate mofetil, and prednisone. Her immediate posttransplant course was complicated by delayed renal graft function, as well as delirium and tremors thought to be associated with uremia and excessive levels of tacrolimus. The delirium and tremors resolved with improved graft function, and her immunosuppression regimen was changed to include cyclosporine rather than tacrolimus. Two months after transplantation, she experienced low-grade fever, fatigue, and headache. Testing for infectious causes, including herpesviruses, enterovirus, West Nile virus, *Borrelia burgdorferi*, and *Cryptococcus*, were negative. Mycophenolate mofetil, valganciclovir, and trimethoprim/sulfamethoxazole prophylaxis were temporarily discontinued because of leukopenia. A week later, she experienced continuing fevers up to 102°F; worsening frontal headache; fatigue; pain in her shins, wrists, and elbows; cognitive slowing; prominent myoclonus; expressive aphasia; and visual hallucinations. She was markedly leukopenic; absolute neutrophil count was 430 cells/μL. Brain magnetic resonance imaging did not reveal mass lesions, infarct, or hydrocephalus. Cerebrospinal fluid testing revealed 17 leukocytes/μL and a protein level of 80.4 mg/dL; however, results of extensive testing for an infectious etiology were negative (Table 1). Routine endomyocardial biopsy samples showed no evidence of rejection; a renal allograft biopsy sample showed evidence of Banff type IIa acute cellular rejection, for which she received 3 doses of methylprednisolone.

After CDC communicated the finding of microsporidiosis in the deceased recipient's allograft, this recipient empirically received treatment with albendazole at 400 mg twice daily. *E. cuniculi* was detected by PCR from urine obtained at the time of albendazole initiation. Her neurologic symptoms resolved, and after 4 months of albendazole therapy, PCR of urine for *E. cuniculi* was negative. Therapy with albendazole was continued for 1 year. As of July 2016, she remained well without any symptoms of microsporidial infection 1 year after stopping albendazole therapy.

Liver Recipient

The liver recipient was a man with hepatitis C–associated cirrhosis and hepatocellular carcinoma. At the time of transplantation, he received intravenous methylprednisolone to induce immunosuppression; at the time of discharge, he received tacrolimus, prednisone, and mycophenolate mofetil to maintain immunosuppression. Thirteen weeks after transplant, the patient visited an outpatient clinic and reported bilateral upper extremity tremor, which was thought to result from elevated tacrolimus levels. A week later, the patient was readmitted to the hospital after an outpatient visit for right lower extremity swelling and shortness of breath; the ultimate diagnosis was deep vein thrombosis and pulmonary embolism. During this hospitalization, the recipient reported lightheadedness, headache, blurry vision, and continued tremor. Because of his neurologic symptoms and identification of microsporidiosis in the deceased right kidney recipient, this patient was also empirically given albendazole. The recipient declined lumbar puncture. Subsequently, microsporidia were identified in a urine specimen by trichrome staining performed at the patient's local hospital (Figure 3, panel A). PCR of the urine specimen confirmed infection with *E. cuniculi*. Microsporidia were also isolated from the urine specimen at CDC after 2 months of cell culture (Figure 3, panel B), and identification was confirmed by IHC and transmission electron microscopy (Figure 3, panel C). The patient's neurologic symptoms resolved, and subsequent urine PCR for *E. cuniculi* was negative. Because of financial constraints, albendazole was discontinued after 4 months of therapy. Repeated PCRs of urine over the next year remained negative for microsporidia. As of December 2015 (14 months after stopping albendazole), the patient remained well without symptoms of microsporidial infection.

Organ Donor

The donor was a middle-aged woman, originally from Mexico but a resident of the United States for several decades. In December 2013, she experienced recurrent and persistent headache, which she attributed to a known history of migraine. The headaches persisted and progressed

Table 2. Transplant recipient testing for microsporidiosis, by specimen and test type*

Patient	Specimen	IHC	TEM	PCR†	Culture
Right kidney recipient	Renal allograft	+	+	+	Not performed
	CNS tissue‡	+	+	Not performed	Not performed
Left kidney/heart recipient	Renal allograft	–	Not performed	+	Not performed
	Cardiac allograft	Indeterminate	-	–	Not performed
	Bone marrow	–	Not performed	–	Not performed
	Urine	Not performed	Not performed	+	Not performed
Liver recipient	Urine	+	+	+	+

*CNS, central nervous system; IHC, immunohistochemistry; TEM, transmission electron microscopy; +, positive; –, negative.
†All PCR positive tests confirmed *Encephalitozoon cuniculi.*
‡CNS tissue included cortex and brainstem.

over several weeks to include nausea, pulsatile tinnitus, and diplopia. Approximately 4 weeks later, she sought care at an emergency department, where brain computed tomography and magnetic resonance imaging revealed several arteriovenous malformations and a right internal carotid saccular aneurysm. She underwent endovascular repair with stenting and coil embolization. This repair was complicated by several episodes of intracranial hemorrhage requiring craniotomy. Despite surgical intervention, her neurologic function continued to decline, and she was eventually declared brain dead. No other symptoms were reported before organ donation. Review of her medical record revealed no reports of gastrointestinal illness during her hospitalization. Interviews with next of kin revealed no clear risk factors for microsporidiosis (e.g., exposure to potentially contaminated water, travel to areas with potentially contaminated drinking water), no gastrointestinal illness before her death, and no travel outside the United States within the previous 12 months.

Because no autopsy was performed on the donor, the only donor specimen available for retrospective testing was archived serum. This sample was tested by microsporidia IFA; a positive titer of 1:2,048 suggested active infection (*13*,*14*).

Discussion

Microsporidia are a diverse group of intracellular, spore-forming organisms; their molecular taxonomic classification is fungi. Microsporidiosis classically occurred in patients with advanced HIV infection before the era of antiretroviral therapy, and it has increasingly occurred in solid organ and hematopoietic stem cell transplant recipients (*15*–*18*). We describe a cluster of 3 solid organ transplant recipients in whom disseminated microsporidiosis developed, the organism having been transmitted by 1 infected donor. Although a laboratory-confirmed donor-derived cluster of microsporidiosis has been reported (*7*), the cluster reported here is unique in that all 3 recipients experienced neurologic disease in the absence of gastrointestinal signs and symptoms, an extremely rare presentation of disseminated microsporidiosis. Previously, CDC has described infection with West Nile virus, rabies, lymphocytic choriomeningitis virus, and *Balamuthia mandrillaris*, transmitted through solid organ transplantation and manifested as encephalitis among recipients (*19*–*22*). The cluster reported here adds microsporidiosis to the list and points to the need for clinicians to maintain awareness of this pathogen when evaluating transplant recipients who exhibit signs or symptoms suggestive of encephalitis.

The most common presentation of microsporidiosis is gastrointestinal disease causing diarrhea and malabsorption; disseminated disease often involves the urinary tract. *E. cuniculi* has been found in the CNS during postmortem examination of patients who had widely disseminated disease, often without clear preceding neurologic manifestations (*23*–*26*). However, presentation with isolated severe neurologic disease, as seen in the transplant recipients in this cluster, is very rare, although it has occurred in immunocompetent persons (*27*,*28*). This rare presentation probably led to the diagnostic and treatment delays for the patients in this cluster. Nonspecific symptoms, such as weakness and confusion, as seen early in the deceased right kidney recipient in this cluster, may also be difficult to ascribe to an infectious or donor-derived etiology, especially for older recipients with multiple concurrent conditions.

Diagnosis of microsporidiosis is challenging; the organisms are not easily visible with routine Gram staining and do not grow in standard culture media. However, if clinical suspicion exists, rapid diagnosis can be accomplished at clinical laboratories by light microscopy of specimens (usually urine or feces) and use of a modified trichrome stain (Figure 3, panel A). Therefore, diagnosis is dependent on clinicians maintaining a high level of suspicion and obtaining the appropriate local testing or referring samples to specialized institutions that can perform advanced diagnostics, such as PCR or cell culture (Figure 3, panel B).

In the cluster we report, an extensive evaluation to determine the etiology of illness was performed for the 2 recipients hospitalized with encephalitis. Microsporidiosis was not considered during the 6-week disease course until an autopsy had been performed on the deceased recipient and subsequent specialized studies at CDC revealed the diagnosis. Clinicians should be aware that the clinical presentation of disseminated microsporidiosis can be a neurologic syndrome in the absence of gastrointestinal

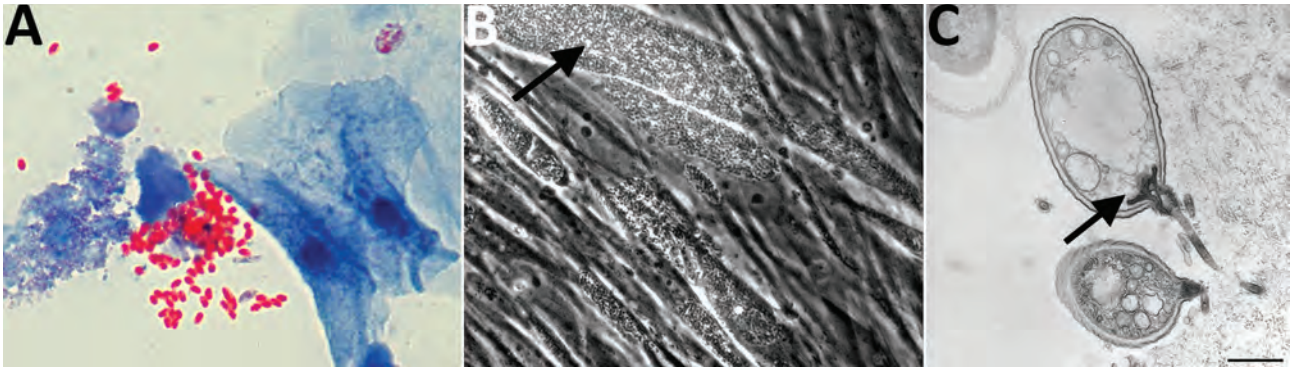

Figure 3. Microsporidia identified in urine samples from liver recipient. A) Urine trichrome stain. Original magnification ×100. B) Cell culture showing microsporidium (arrow). Original magnification ×200. C) Transmission electron microscopic image of infected cell culture with germinating microsporidial spore (arrow). Scale bar indicates 500 nm.

symptoms. Further research is needed to understand whether a neurotropic variant of *E. cuniculi* might be the cause of isolated severe neurologic microsporidiosis, as occurred in these 3 patients. Furthermore, given that animal studies have shown that encephalitozoonosis can cause vasculitic manifestations, including aortitis (*29*) and artertitis (*30*) in primates, the intracerebral pathology of the donor might have been associated with occult disseminated *E. cuniculi* infection.

Although microsporidial infections have been described for transplant patients, establishing a donor-derived etiology is challenging for many reasons. Microsporidia are transmitted by the fecal–oral route; thus, any exposure to feces, either directly or via contaminated water or soil, probably represents a mode of acquisition. However, little is known about additional risk factors or other ways of acquiring microsoporidial infection. Similarly, little data are available on incubation period, duration of illness/shedding, and likelihood of dissemination in immunocompetent or immunocompromised patients. Therefore, it may not be possible to determine the source of infection in transplant recipients (e.g., derived from transplant vs. acquired from the environment) and the likelihood of underlying donor infection on the basis of clinical course or characteristics alone. However, when a similar infectious syndrome develops in ≥2 recipients of organs from a common donor, the chance that it is donor-derived increases substantially. After donor-derived infection is suspected, assessing whether a donor had active disseminated or even clinically significant microsporidial infection is also challenging. Although organ procurement organizations are required to keep donor serum samples for postmortem testing, there is no commercially available serologic test for microsporidiosis. IFA testing, along with advanced testing to compare species, as was used in this investigation, require reference laboratory capacity. Although donor disease can be identified through examination of tissues, autopsies are frequently not conducted, and archived tissues are often unavailable. Identification of new and emerging donor-derived infections could be greatly facilitated if donor autopsy rates are increased.

Microsporidia species identification has treatment implications. Although Encephalitozoonidae are susceptible to albendazole, *Enterocytozoon bieneusi*, another common species of microsporidia affecting humans, is not. Although immunocompetent persons may require no treatment or short-course therapy (*31*,*32*), the appropriate treatment duration for immunocompromised patients remains unclear. For patients with HIV/AIDS, immune reconstitution is a key component to treatment. However, in transplant recipients for whom de-escalation of immunosuppression may not be immediately possible, the optimal length of treatment has not been well studied. Although some recommendations suggest 2–4 weeks of therapy, the patients described in this cluster received treatment until demonstration of clearance of infection (as identified through urine PCR). Unfortunately, despite having recently received a resource-intensive intervention such as liver transplant, the expense associated with albendazole (reportedly ≈$2,000/month), a drug that has been available in generic form since the 1980s and is on the World Health Organization Essential Medicine List (*33*), precluded continuing therapy for the liver recipient. High costs of generic drugs with niche markets have been linked to drug shortages, supply disruptions, or, in the case of generic albendazole, consolidations in the generic drug industry, leading to a single manufacturer with the ability to set prices without competition (*34*).

The findings of this investigation are subject to limitations. Because no donor tissue was available for testing, we were unable to differentiate whether the donor's neurologic complaints and radiographic findings were caused by microsporidial disease or whether she had subclinical disseminated infection. Additionally, the IFA result interpretation has not been standardized for the serologic diagnosis of microsporidiosis. Last, sequencing of *E. cuniculi*

strains (e.g., the internal transcribed spacer region of rRNA or whole-genome sequencing) was not performed as part of the public health investigation, and we are thus unable to further investigate strain relatedness. However, we were able to confirm infection with the same species of microsporidia in all 3 recipients and the donor, which is highly suggestive of donor-derived transmission.

Identifying donor-derived disease transmission events and ensuring appropriate treatment and management of recipients requires extensive collaboration. This investigation, as did other donor-derived disease transmissions described by CDC, required close cooperation among clinicians, laboratory scientists, the organ procurement organization, and public health agencies. Clinicians should maintain a high index of suspicion for donor-derived infections and should report any suspected or potential events to the Organ Procurement and Transplantation Network.

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Epidemiology of Invasive *Haemophilus influenzae* Disease, Europe, 2007–2014

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We describe the epidemiology of invasive *Haemophilus influenzae* disease during 2007–2014 in 12 European countries and assess overall *H. influenzae* disease trends by serotype and patient age. Mean annual notification rate was 0.6 cases/100,000 population, with an increasing annual trend of 3.3% (95% CI 2.3% to 4.3%). The notification rate was highest for patients <1 month of age (23.4 cases/100,000 population). Nontypeable *H. influenzae* (NTHi) caused 78% of all cases and showed increasing trends among persons <1 month and ≥20 years of age. Serotype f cases showed an increasing trend among persons ≥60 years of age. Serotype b cases showed decreasing trends among persons 1–5 months, 1–4 years, and ≥40 years of age. Sustained success of routine *H. influenzae* serotype b vaccination is evident. Surveillance systems must adopt a broad focus for invasive *H. influenzae* disease. Increasing reports of NTHi, particularly among neonates, highlight the potential benefit of a vaccine against NTHi.

Haemophilus influenzae, a pleomorphic gram-negative coccobacillus, is a common commensal of the upper respiratory tract. It is a human-only pathogen that can cause severe invasive disease, including meningitis, pneumonia, and septicemia. *H. influenzae* strains are divided based on the presence or absence of a polysaccharide capsule; there are 6 encapsulated serotypes (*H. influenzae* serotypes a [Hia], b [Hib], c [Hic], d [Hid], e [Hie], and f [Hif]) and nonencapsulated, nontypeable *H. influenzae* (NTHi) strains. Although Hib strains are considered the most pathogenic, NTHi accounts for a high proportion of all *H. influenzae* infections because it causes a notable number of noninvasive infections, such as otitis media and sinusitis, as well as invasive infections (*1*–*4*).

Beginning in 1989, countries of the European Union and European Economic Area (EU/EEA) began introducing conjugate Hib vaccination into their routine national immunization programs; most countries introduced the vaccine before the year 2000. In the prevaccine era, Hib was estimated to cause most cases of invasive *H. influenzae* disease and was a leading cause of bacterial meningitis worldwide, primarily among otherwise healthy children <5 years of age (*5*,*6*). The introduction of Hib vaccine has led to a substantial and sustained reduction in infection caused by Hib (*7*–*12*) and in pharyngeal Hib carriage, resulting in herd protection (*8*,*13*,*14*). The World Health Organization recommends the inclusion of Hib vaccination in all routine infant immunization programs as a 3-dose primary schedule with or without a booster dose or as a 2-dose primary schedule with a booster dose (*15*). Since 2010, Hib vaccination has been part of the national immunization program in all EU/EEA countries, and high coverage has been maintained (*16*). Following the introduction of Hib vaccine, several studies in Europe and elsewhere reported increasing trends in NTHi, Hia, Hie, and Hif infections (*3*,*4*,*7*,*17*,*18*), and NTHi is now the leading cause of invasive *H. influenzae* disease in EU/EEA countries and other areas worldwide (*2*–*4*). Most studies do not report evidence of strain replacement due to Hib vaccine introduction, although some have supported this occurrence (*7*–*9*,*11*,*17*,*19*–*21*).

In 1996, the European Union Invasive Bacterial Infections Surveillance Network began Europe-level surveillance of invasive *H. influenzae* disease, and since 2007, surveillance has been coordinated by the European Centre for Disease Prevention and Control (ECDC) (*7*). We conducted a study to describe the epidemiology of invasive *H. influenzae* disease in EU/EEA countries during 2007–2014 and to monitor age- and serotype-specific trends during the study period.

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Methods

European Surveillance of Invasive *H. influenzae* Disease

On an annual basis, all 28 EU Member States and 2 EEA countries report national surveillance data on invasive *H. influenzae* disease to a central database at ECDC. Most of the 30 reporting countries provide data from passive surveillance systems, including mandatory reporting, that cover their entire national populations (*22*). All 30 countries report using the EU case definition for invasive *H. influenzae* (*23*) or a case definition with compatible criteria for laboratory confirmation of disease. Invasive *H. influenzae* disease is confirmed by isolation of *H. influenzae* from a normally sterile site; culture is used for confirmation of >99% of all reported cases. According to the most recent external quality assurance scheme run by the ECDC-funded IBD-labnet (the invasive bacterial disease laboratory surveillance network in Europe), 20 countries also use a PCR-based method to confirm species identity. Twenty-eight countries routinely serotype isolates, most by slide agglutination, PCR, or both methods (*24*).

Data Selection and Preparation

We analyzed data on invasive *H. influenzae* disease reported to ECDC during 2007–2014. We excluded cases not reported as laboratory-confirmed or for patients with unreported age or sex. We excluded data from countries that 1) had not reported case-based data for all years in the study period; 2) had introduced Hib vaccination into their national immunization program during the study period; 3) had reported ≥50% of cases as meningitis, Hib, or both, which may indicate a surveillance bias toward the reporting of these cases; or 4) had not reported serotype data for all years and/or had reported serotype data for <50% of cases.

We used surveillance system coverage data and population data from Eurostat (http://www.ec.europa.eu/eurostat) as denominators for calculating the total and age-specific notification rates per 100,000 population. We categorized data on age into the following patient age groups; <1, 1–4, 5–19, 20–39, 40–59, and ≥60 years of age. We further categorized the infant (<1 year of age) age group into <1 month, 1–5 months, and 6–11 months of age. We estimated the denominator in these infant age groups as the total infant population divided by 12 and multiplied by the number of months in each age group. Countries that did not report data on the age of infants in months were excluded from the analysis of infant age groups.

Data Analysis

We described the epidemiology of invasive *H. influenzae* disease by year, country, and serotype and by patient age group, sex, and clinical presentation. We compared patient age distributions by *H. influenzae* serotype by calculating median ages with interquartile ranges and comparing them using the Kruskal-Wallis test. The Dunn test was used to perform post hoc pairwise multiple comparisons. We used male:female notification rate ratios to describe the sex distribution of patients by age group, serotype, or both. We applied Poisson regression models to estimate differences in male and female notification rates and male:female notification rate ratios. We expressed categorical variables as the number of cases and proportion (%) and compared them using the χ^2 test.

We assessed overall temporal trends by estimating the percentage change in annual notification rates, including 95% CIs, by age group, serotype, or both by using linear regression analysis of the log of the annual notification rate. We used reporting country as a cluster effect in the models. We fixed the significance level at $p = 0.05$ and used Stata 14 (StataCorp LLC, College Station, TX, USA) to analyze data.

Results

We included data from 12 of the 30 EU/EEA countries: Belgium, Cyprus, the Czech Republic, Denmark, Finland, Ireland, Italy, the Netherlands, Norway, Slovenia, Spain, and the United Kingdom. Belgium and Spain had voluntary reporting, but the other countries had mandatory reporting. Belgium and the Czech Republic described their surveillance system as active; all other countries reported having passive surveillance systems. Surveillance system population coverage was 50% in Spain and 100% in the other 11 countries. Together, the surveillance systems in these 12 countries covered 41% of the total EU/EEA population. The year of Hib vaccine introduction in the 12 countries ranged from 1992 to 2001. With 1 exception, 3-dose vaccination coverage was >90% in all countries during the study period; Denmark had 87%–89% coverage during 2007–2009 (*16*).

Of the remaining 18 EU/EEA countries, we excluded 4 for not reporting case-based data for all study years (Bulgaria, Croatia, Luxembourg, Romania) and 2 for introducing the vaccine during the study period (Bulgaria, Poland). We also excluded 5 countries for reporting ≥50% of cases as meningitis or Hib (Estonia, Greece, Hungary, Latvia, Slovakia), and we excluded 8 for not reporting serotype data for all years, reporting serotype data for <50% of cases, or both (Austria, France, Germany, Iceland, Lithuania, Malta, Portugal, Sweden).

During 2007–2014, the 12 countries included in the study reported a total of 10,624 cases of invasive *H. influenzae* disease for a mean annual notification rate of 0.6 cases/100,000 population. The overall notification rate increased 3.3% (95% CI 2.3% to 4.3%) annually during the study period (Table 1). By country, the notification rate ranged from 1.6 cases/100,000 population (n = 637) in Norway to 0.1 case/100,000 population (n = 6) in Cyprus

Table 1. Epidemiologic findings for cases of invasive *Haemophilus influenzae* disease, by patient age group and year of notification, in 12 countries in Europe, 2007–2014*

Age group	Annual notification rate/100,000 population (no. cases)								Mean annual notification rate (no. cases)	M:F ratio	% Change in annual notification rate (95% CI)†
	2007	2008	2009	2010	2011	2012	2013	2014			
<1 y	4.3 (96)	4.4 (101)	5.4 (125)	4.4 (103)	5.2 (120)	4.7 (106)	4.3 (97)	6.5 (140)	4.9 (888)	1.24	2.8 (–2.1 to 8.0)
<1 mo‡	17.5 (29)	21.3 (36)	24.8 (43)	23.2 (40)	25.5 (44)	20.0 (34)	24.3 (41)	30.8 (51)	23.4 (318)	1.04	5.0 (–0.2 to 10.4)
1–5 mo‡	4.5 (37)	3.3 (28)	3.8 (33)	3.0 (26)	3.4 (29)	2.9 (25)	1.9 (16)	3.0 (25)	3.2 (219)	1.75	–7.1 (–13.3 to 0.4)
6–11 mo‡	2.3 (23)	3.0 (30)	3.6 (37)	1.8 (19)	2.7 (28)	3.3 (34)	2.8 (28)	4.5 (45)	3.0 (244)	1.16	6.0 (–4.3 to 16.5)
1–4 y	1.1 (97)	0.9 (80)	1.0 (88)	0.7 (67)	0.9 (80)	0.7 (62)	0.9 (85)	1.0 (93)	0.9 (652)	1.25	–2.01 (–8.3 to 4.6)
5–19 y	0.2 (58)	0.2 (63)	0.2 (61)	0.1 (45)	0.2 (63)	0.1 (44)	0.2 (57)	0.2 (71)	0.2 (462)	1.29	0.3 (–6.3 to 7.3)
20–39 y	0.2 (113)	0.2 (118)	0.2 (111)	0.2 (134)	0.3 (139)	0.2 (114)	0.2 (115)	0.3 (148)	0.2 (992)	0.56	2.8 (–1.2 to 6.9)
40–59 y	0.3 (190)	0.4 (204)	0.4 (209)	0.4 (201)	0.4 (255)	0.4 (206)	0.40 (228)	0.4 (230)	0.4 (1,723)	0.98	1.5 (–1.4 to 4.6)
>60 y	1.3 (606)	1.4 (638)	1.4 (675)	1.5 (703)	1.5 (727)	1.7 (848)	1.7 (836)	1.7 (874)	1.5 (5,907)	1.28	**3.8 (2.5 to 5.1)**
Total§	0.6 (1,160)	0.6 (1,204)	0.6 (1,269)	0.6 (1,253)	0.7 (1,384)	0.7 (1,380)	0.7 (1,418)	0.7 (1,556)	0.6 (10,624)	1.05	**3.3 (2.3 to 4.3)**

*The study was conducted in Belgium, Cyprus, the Czech Republic, Denmark, Finland, Ireland, Italy, the Netherlands, Norway, Slovenia, Spain and the United Kingdom. Data are for a total of 10,624 cases.
†Bold font indicates statistically significant trends (p = 0.05).
‡For these age groups, data from only 11 countries are included because Spain did not report data on the age of infant cases by month.
§Totals do not include data separately shown for infants <1 mo, 1–5 mo, and 6–11 mo of age because those data are included in the <1 y age group.

(Figure 1). We observed increasing overall trends in Denmark, Italy, the Netherlands, and Spain and insignificant trends in all other countries.

Age and Sex of Case-Patients

Of the 10,624 case-patients, 5,907 (56%) were ≥60 years of age, and 888 (8.4%) were <1 year of age (Table 1). The notification rate was highest for infants (4.9 cases/100,000 population), followed by persons ≥60 years of age (1.5/100,000). The notification rate among persons ≥60 years of age increased 3.8% (95% CI 2.5% to 5.1%) annually. The age in months was available for 781 (88%) of the 888 infants; Spain was the only country not to report any data on month of age. The notification rate for infants <1 month of age (23.4 cases/100,000 population) was >7-fold higher than that for those 1–5 months of age (3.2/100,000)

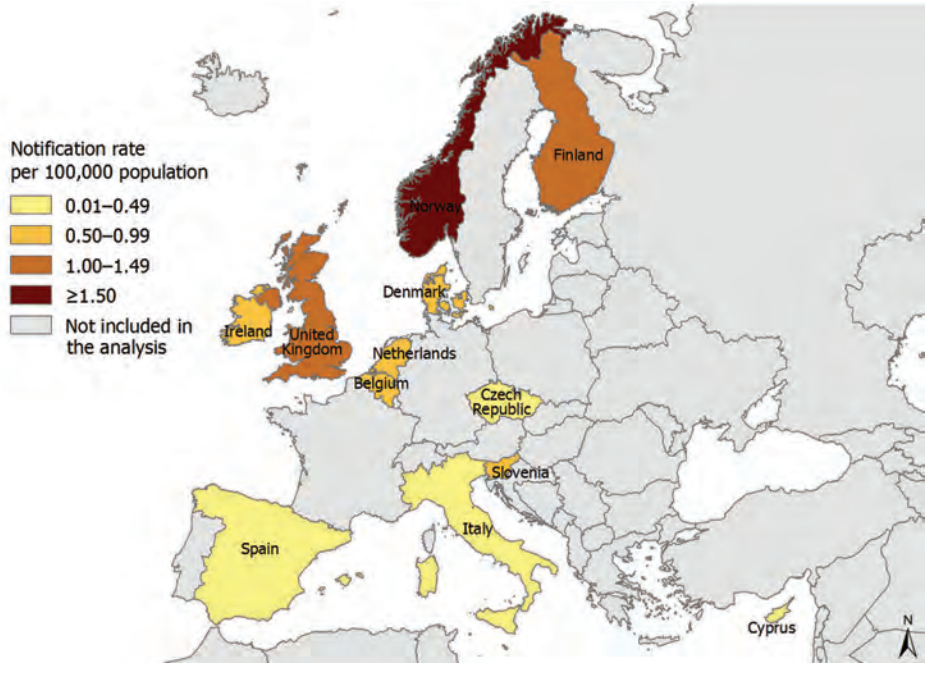

Figure 1. Notification rate for cases of invasive of *Haemophilus influenzae* disease in 12 European countries, 2007–2014. A total of 10,624 cases were notified.

and 6–11 months of age (3.0/100,000). The notification rate for infants 1–5 months of age decreased 7.1% (95% CI −13.3% to −0.4%) annually. The overall male:female notification rate ratio was 1.05 (95% CI 1.01 to 1.09) (Table 1).

Serotype

H. influenzae serotype was reported for 8,781 (83%) of the 10,624 patients (Table 2). The age distribution did not differ between case-patients with serotype reported and those with serotype not reported (p = 0.319). Case-patients without a reported serotype were more likely than those with a reported serotype to be male (male:female notification rate ratio 1.20 vs. 0.97, respectively; p = 0.001).

A total of 6,853 (78%) of the 8,781 cases with a reported serotype were caused by NTHi strains; these strains also accounted for most cases in all age groups (Table 2). The notification rate for NTHi cases was highest among infants and persons ≥60 years of age; most cases were in the older age group. We observed this same notification profile among Hie (239/8,781 [3%]) and Hif (828/8,781 [9%]) cases. Case-patients with Hib infection (811/8,781 [9%]) had a lower median age than those with Hie (p<0.001), Hif (p<0.001), or NTHi (p<0.001) infection. Hib caused 19% (250/1,343) of all cases among children <5 years of age and had highest notification rates among infants and children 1–4 years of age. However, most Hib cases were in persons ≥40 years of age (Table 2). *H. influenzae* serotype was reported for 86% (673/781) of infants with known month of age. NTHi caused most cases in all infant age groups; most notably, NTHi caused 97% (263/271) of cases among infants <1 month of age (a notification rate of 19.4 cases/100,000 population) (Table 2).

Among 20- to 39-year-old patients, more women than men were infected with Hie (male:female notification rate ratio 0.09, 95% CI 0.11 to 0.69), Hif (0.55, 95% CI 0.31 to 0.99), and NTHi (0.44, 95% CI 0.38 to 0.53). Conversely, among patients ≥60 years of age, more men than women were infected by Hie (1.45, 95% CI 1.06 to 1.99) and NTHi (1.30, 95% CI 1.22 to 1.38), and more boys than girls were infected by NTHi among children <1 year of age (1.20, 95% CI 1.02 to 1.42) and 1–4 years of age (1.37, 95% CI 1.11 to 1.69).

The notification rate of NTHi cases increased 7.4% (95% CI 5.3% to 9.6%) annually, driven by increasing trends in NTHi cases among children <1 year of age and persons ≥20 years of age. The increasing trend in infants was driven by a 6.2% (95% CI 2.8% to 9.8%) annual increase in the notification rate among those <1 month of age (Table 3). The notification rate of Hib cases decreased

Table 2. Mean annual notification rate per 100,000 population and number of cases of invasive *Haemophilus influenzae* disease by serotype and patient age group, in 12 European countries, 2007–2014*

	Mean annual notification rate/100,000 population, by serotype (no. cases)†										Mean overall annual notification rate (no. cases)
Variable	Hia	Hib	Hic	Hid	Hie	Hif	NTHi	Non-b	Total with data	Unknown	
Age group											
<1 y	0.05 (9)	0.65 (118)	0	0	0.06 (11)	0.30 (54)	3.17 (578)	0.005 (1)	4.24 (771)	0.64 (117)	4.90 (888)
<1 mo‡	0.07 (1)	0.29 (4)	0	0	0 (0)	0.22 (3)	19.37 (263)	0	19.96 (271)	3.46 (47)	23.42 (318)
1–5 mo‡	0.01 (1)	0.65 (44)	0	0	0.06 (4)	0.22 (15)	1.75 (119)	0.01 (1)	2.71 (184)	0.52 (35)	3.23 (219)
6–11 mo‡	0.09 (7)	0.74 (60)	0	0	0.09 (7)	0.33 (27)	1.44 (117)	0	2.68 (218)	0.32 (26)	3.00 (244)
1–4 y	0.006 (4)	0.18 (132)	0.001 (1)	0.003 (2)	0.01 (8)	0.07 (52)	0.51 (369)	0.006 (4)	0.79 (572)	0.11 (80)	0.90 (652)
5–19 y	<0.001 (1)	0.02 (54)	<0.001 (1)	0	0.003 (9)	0.01 (26)	0.10 (277)	<0.001 (1)	0.14 (369)	0.03 (93)	0.17 (462)
20–39 y	<0.001 (1)	0.02 (71)	0	<0.001 (1)	0.003 (12)	0.01 (50)	0.15 (648)	<0.001 (2)	0.18 (785)	0.05 (207)	0.22 (992)
40–59 y	<0.001 (4)	0.04 (189)	0	<0.001 (1)	0.009 (41)	0.03 (153)	0.21 (980)	<0.001 (1)	0.30 (1,369)	0.08 (354)	0.37 (1,723)
>60 y	0.001 (4)	0.06 (247)	0	<0.001 (6)	0.04 (158)	0.13 (493)	1.03 (4,001)	<0.001 (6)	1.27 (4,915)	0.26 (992)	1.53 (5,907)
Overall notification rate (no. cases)§	0.001 (23)	0.05 (811)	<0.001 (2)	<0.001 (10)	0.01 (239)	0.05 (828)	0.42 (6,853)	<0.001 (15)	0.53 (8,781)	0.11 (1,843)	0.64 (10,624)
Median age, y§	2	43	3	69	66	64	65	32	64	62	63
IQR, y§	0–55	3–63	1–5	33–76	53–78	45–75	35–79	2–83	33–78	37–76	34–77

*The study was conducted in Belgium, Cyprus, the Czech Republic, Denmark, Finland, Ireland, Italy, the Netherlands, Norway, Slovenia, Spain, and the United Kingdom. Data are for a total of 10,624 cases. Hia, *H. influenzae* serotype a; Hib, serotype b; Hic, serotype c; Hid, serotype d; Hie, serotype e; Hif, serotype f; NTHi, nontypeable *H. influenzae*; non-b, cases reported as a non-b *H. influenzae* strain (It was not known whether these cases were encapsulated); IQR, interquartile range.

†Values are mean annual notification rate/100,000 population (no. cases), except as indicated.

‡For these age groups, data from only 11 countries are included because Spain did not report data on the age of infant cases by month.

§Totals do not include data separately shown for infants <1 mo, 1–5 mo, and 6–11 mo of age because those data are included in the <1 y patient age group.

11.9% (95% CI –16.0% to –7.5%) annually, driven by decreasing trends in Hib cases among persons <1 year, 1–4 years, 40–59 years, and ≤60 years of age (Figure 2; Table 3). The decreasing trend in infants was driven by a 25.0% (95% CI –32.2% to –17.0%) annual decrease in cases among infants 1–5 months of age (Table 3). No significant overall trend was observed among Hie or Hif cases or collectively among cases caused by encapsulated serotypes Hia–Hif (Figure 2; Table 3). The notification rate of Hie cases among children 1–4 years of age decreased 14.2% (95% CI –25.0% to –1.7%) annually (Table 3), although only 8 cases were reported for this serotype and age group during the study period (Table 2). The notification rate of Hif cases among persons ≥60 years of age increased 7.0% (95% CI 0.9% to 13.4%) annually (Table 3). Each year during 2010–2014, more cases of Hif than Hib were reported (Figure 2). Too few cases of Hia, Hic, and Hid were reported to calculate trends for these serotypes (Table 2). The notification rate of cases reported with unknown serotype decreased 4.8% (95% CI –9.0% to –0.5%) annually (Figure 2; Table 3).

By country, an 18.5% (95% CI 1.9% to 37.9%) increasing trend in Hib was observed in Italy, although only 26 cases were reported during the study period, and no more than 5 cases were reported in a single year. The notification rate did not increase significantly for any encapsulated serotype in any other country. The notification rate for NTHi cases increased significantly in Belgium, Denmark, Ireland, Italy, the Netherlands, Spain, and the United Kingdom (data not shown). In all other countries, the change in the NTHi notification rate over the study period was not significant.

Clinical Presentation

Clinical presentation was known for 6,722 (63%) of the reported 10,624 case-patients. Most had septicemia (4,128 patients [61%]), bacterial pneumonia (1,207 [18%]), or meningitis (596 [9%]). The following clinical presentations were also reported: osteomyelitis (75 patients [1%]), meningitis and septicemia (64 [1%]), epiglottitis (52 [1%]), and cellulitis (37 [1%]), and other (563 [8%]). Septicemia was the most common clinical presentation in all age groups.

Clinical presentation was known for 5,913 (67%) of the 8,781 patients with serotyped isolates. For all the different clinical presentations, except epiglottitis, NTHi was the most common cause of *H. influenzae* infection; 78% of cases presenting with epiglottitis were caused by Hib. Septicemia was reported for most cases caused by Hib (51%), Hie (67%), Hif (61%), and NTHi (66%), and it was the most common clinical presentation for all age groups infected with these serotypes, except infants infected with Hie and Hif (60% and 45%, respectively, were reported to have meningitis) (Figure 3). Bacterial pneumonia was most prominent among older age groups with Hib, Hie, and Hif infection, but it was observed across all age groups with NTHi infection (Figure 3). Among 212 infants <1 month of age with available clinical presentation and serotype data, 181 (85%) had NTHi infection presenting with septicemia.

Discussion

The sustained low notification rate for Hib and continued decreasing infection trend in all age groups (i.e., in those targeted and not targeted for vaccination) underscore the success of routine Hib vaccination. Among children <5 years of age with invasive *H. influenzae* disease, almost 1 in 5 cases is still caused by Hib, a potentially preventable disease. Breakthrough cases of invasive disease following Hib vaccination have been reported in immunocompromised and healthy children (*25*,*26*); however, vaccine failures are rare, and additional vaccine doses have are an effective way to achieve protective antibody levels in such instances (*25*). Although Hib vaccination has notably

Table 3. Percentage change in annual notification rate for cases of invasive *Haemophilus influenzae* disease, by serotype and patient age group, in 12 European countries, 2007–2014*

Age group	% Change (95% CI), N = 10,574†				
	Hib	Hie	Hif	NTHi	Unknown serotype
<1 y	**–8.5 (–14.5 to –2.1)**	–4.3 (–33.6 to 38.1)	–6.2 (–23.6 to 15.2)	**5.5 (0.6 to 10.8)**	4.1 (–4.1 to 13.0)
<1 mo‡	–	–	–0.9 (–4.3 to 2.6)	**6.2 (2.8 to 9.8)**	–0.3 (–19.3 to 23.1)
1–5 mo‡	**–25.0 (–32.2 to –17.0)**	–2.4 (–72.9 to 251.8)	11.7 (–8.2 to 36.0)	2.1 (–3.3 to 7.7)	–7.1 (–15.6 to 2.4)
6–11 mo‡	3.5 (–18.4 to 31.1)	–	–4.8 (–21.2 to 15.1)	2.7 (–9.7 to 16.8)	24.9 (–2.9 to 60.7)
1–4 y	**–18.4 (–32.9 to –0.8)**	**–14.2 (–25.0 to –1.7)**	10.1 (–8.7 to 32.7)	3.8 (–3.4 to 11.6)	2.8 (–9.3 to 16.5)
5–19 y	–8.3 (–26.2 to 14.1)	–	–0.2 (–26.3 to 35.0)	5.3 (–4.7 to 16.3)	–4.9 (–20.3 to 13.5)
20–39 y	–15.0 (–29.4 to 2.3)	3.4 (–14.8 to 25.5)	–1.4 (–17.6 to 18.0)	**9.7 (5.6 to 13.9)**	**–8.6 (–16.2 to –0.3)**
40–59 y	**–9.0 (–14.7 to –3.0)**	–3.3 (–19.8 to 16.7)	7.0 (–3.8 to 19.0)	**6.8 (2.4 to 11.3)**	**–6.8 (–11.0 to –2.3)**
>60 y	**–12.6 (–17.8 to –7.1)**	12.7 (–2.9 to 30.8)	**7.0 (0.9 to 13.4)**	**7.0 (4.5 to 9.5)**	–6.0 (–12.1 to 0.5)
Total§	**–11.9 (–16.0 to –7.5)**	6.3 (–5.3 to 19.5)	6.4 (–1.5 to 14.8)	**7.4 (5.3 to 9.6)**	**–4.8 (–9.0 to –0.5)**

*The study was conducted in Belgium, Cyprus, the Czech Republic, Denmark, Finland, Ireland, Italy, the Netherlands, Norway, Slovenia, Spain, and the United Kingdom. Data are for a total of 10,574 cases. Hib, *H. influenzae* serotype b; Hie, serotype e; Hif, serotype f; NTHi, nontypeable *H. influenzae*; – no cases reported or no trend could be determined.
†Bold font indicates statistically significant trends (p = 0.05).
‡For these age groups, data from only 11 countries are included because Spain did not report data on the age of infant cases by month.
§Totals do not include data separately shown for infants <1 mo, 1–5 mo, and 6–11 mo of age because those data are included in the <1 y age group.

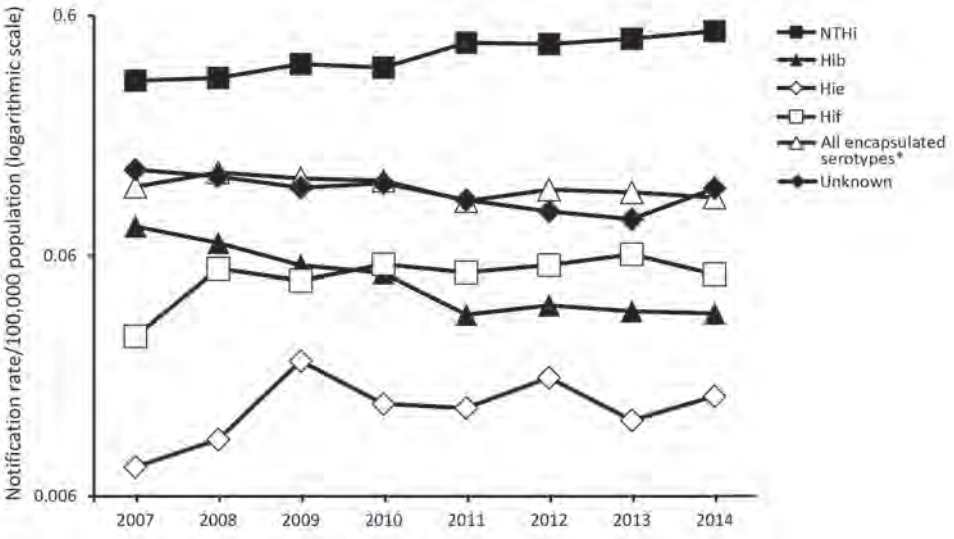

Figure 2. Notification rate for cases of invasive *Haemophilus influenzae* disease, by serotype and year of notification, in 12 countries in Europe, 2007–2014. A total of 8,781 cases were notified. Cases were notified from Belgium, Cyprus, the Czech Republic, Denmark, Finland, Ireland, Italy, the Netherlands, Norway, Slovenia, Spain, and the United Kingdom. *Refers to all cases reported as *H. influenzae* serotypes a (Hia), b (Hib), c (Hic), d (Hid), e (Hie), and f (Hif).

decreased the incidence of invasive Hib disease in all age groups, this reduction has been greatest among young children (*3*,*10*,*11*,*27*), and most Hib cases now occur in older adults with concurrent conditions (*27*,*28*).

In the prevaccine era, NTHi was not a known common cause of invasive infection (*29*), but it is now well recognized as the leading cause of invasive *H. influenzae* disease (*2*–*4*). Higher *H. influenzae* notification rates for infants, particularly neonates, the elderly, and women of childbearing age, were described before (*30*,*31*) and after (*32*–*34*) the introduction of routine Hib vaccination. In addition, several studies showed an increased burden of NTHi in groups more susceptible to infection, with high proportions of intensive care admission, high case-fatality rates, and frequent sequelae among survivors (*2*,*29*,*32*,*35*). The notification rate of NTHi cases in infants <1 month of age, with most cases presenting as septicemia, is particularly striking. Studies have shown that most cases in neonates are present at the time of birth, and infection may induce labor (*33*), causing premature birth (*3*,*33*,*36*). It is probable that the number of NTHi infections among neonates is underestimated (*37*), although the increasing notification rate among infants <1 month of age indicates that reporting may be improving. If developed, a vaccine against NTHi that could be administered to pregnant women could provide protection to expectant mothers and neonates (*35*). The genetic diversity of NTHi complicates vaccine development, but exploration into potential NTHi vaccine candidates is ongoing (*38*).

The increasing recognition of NTHi as a key invasive pathogen highlights how future surveillance of invasive *H. influenzae* disease must encompass all serotypes and strains, age groups, and clinical presentations. EU/EEA member states are not required to report all *H. influenzae* strains. Moreover, simply studying NTHi trends may now be insufficient for monitoring changes in the epidemiology of NTHi strains because they are more genetically diverse than encapsulated strains (*29*,*35*,*37*,*39*). Surveillance of NTHi in Europe may benefit from more genetic typing studies of circulating strains, with regard to carriage and disease, and the standardization of typing methodologies (*24*,*36*,*37*).

The notification rate of non-Hib encapsulated serotypes in Europe remains low and stable. Some studies have reported increasing trends in Hia cases after the introduction of routine Hib vaccination (*18*,*40*,*41*); however, Hia remains rare in Europe.

We observed increasing trends in the annual notification rate of NTHi cases in persons <1 and ≥20 years of age and of Hif cases in persons ≥60 years of age. These trends may represent an actual increase in the incidence of disease, which could result from different factors, such as population aging and increased use of immunosuppressive therapy, both of which would increase the number of persons at risk for infection by these strains (*17*,*35*). Despite these increasing trends, we could not assess possible strain replacement resulting from the introduction of Hib vaccination because we could not compare serotype distributions or incidence between the prevaccination and postvaccination periods. Trends also may reflect changes and improvements in surveillance that increase case detection, such as an increase in awareness among clinicians since Hib vaccine introduction, changing blood culture practices, and more accurate serotyping techniques. For example, since 1993 in the Netherlands, the reporting of NTHi from blood isolates has increased, while the reporting of NTHi from cerebrospinal fluid isolates has remained stable (*42*). Furthermore, new molecular technologies, such as PCR-based serotyping, have allowed more accurate differentiation between typeable and nontypeable strains (*24*,*37*).

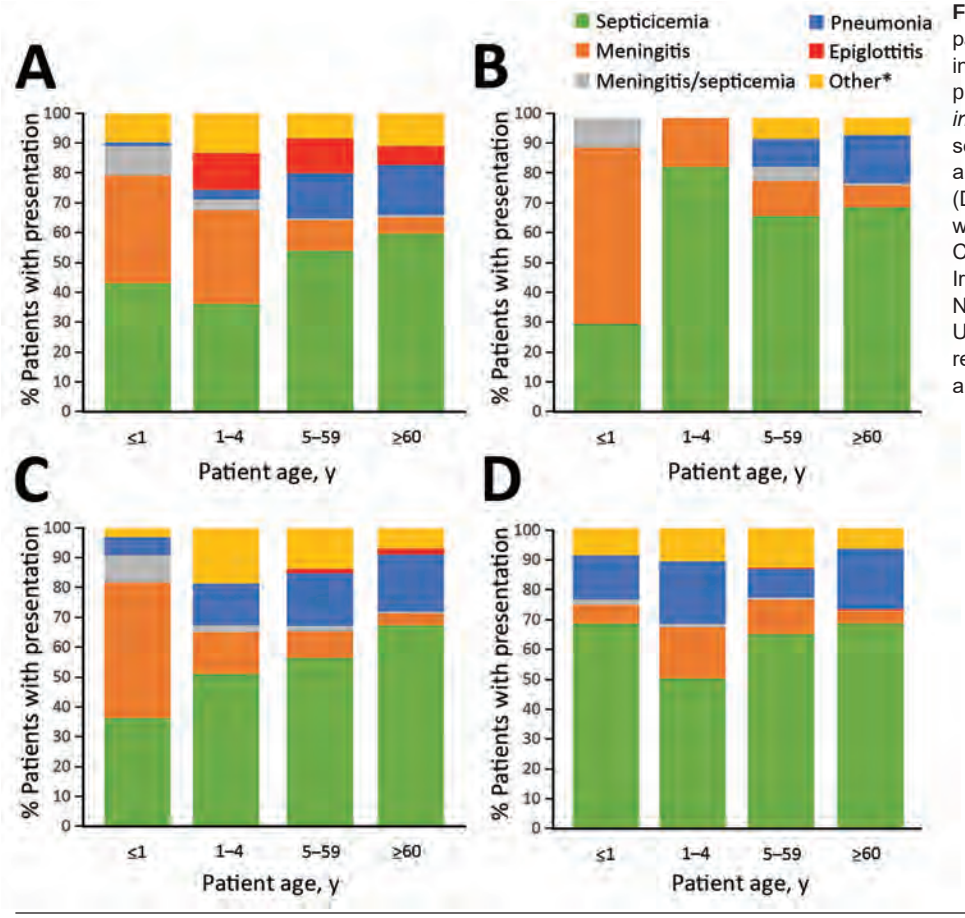

Figure 3. Percentage of cases, by patient age group, in 12 countries in Europe with various clinical presentations of *Haemophilus influenzae* disease caused by serotypes b (A), e (B), and f (C) and by nontypeable *H. influenzae* (D), 2007–2014. Cases (N = 5,879) were in Belgium, Cyprus, the Czech Republic, Denmark, Finland, Ireland, Italy, the Netherlands, Norway, Slovenia, Spain, and the United Kingdom. *Refers to cases reported as other, cellulitis, septic arthritis, or osteomyelitis.

Such technologies are becoming more widely used across the EU/EEA; in 2014, a total of 24 reference laboratories performed PCR-based serotyping, compared with 19 laboratories in 2012 (*24*).

Limitations of our study were the need to combine and compare data from different countries that had possible differences in surveillance sensitivity and methodology and the predisposition for underreporting in routine passive surveillance systems (*43*). The notification rate of invasive *H. influenzae* disease in the United States in 2014, detected through Active Bacterial Core surveillance, was >2 times that of the 12 countries in this study (*44*). Nevertheless, for the entire study period, all included countries used comparable case definitions and reported consistently high quality data for all age groups, serotypes, and clinical presentations, thus indicating no potential surveillance bias. Together, these 12 countries covered 41% of the EU/EEA population, higher than the population coverage in similar large studies (*3*,*7*), and trends observed in each country were consistent with the pooled results for Europe. The surveillance of invasive *H. influenzae* disease on the Europe level is longstanding (*7*) and allows the pooling of data to increase the precision of estimates for what is now a rare disease in the EU/EEA. National reference laboratories in all countries participate in the external quality assurance schemes and training run by IBD-labnet (*24*). Unfortunately, we could not assess specific risk factors, such as concurrent conditions, or sequelae among surviving case-patients because such data are not collected at ECDC. We also could not assess potential vaccine failures because the date of last vaccination was not collected for patients, and the completeness of data regarding the vaccination status of patients with Hib infection was low. In addition, data on fatal outcome were not included because completeness of the data was low. These limitations, along with the fact that data from only 12 of 30 countries were included, underscore the potential for improving the scope and quality of data reported to ECDC and increasing the value of surveillance on the Europe level.

In conclusion, the sustained success of routine Hib vaccination is evident, however the epidemiology of invasive *H. influenzae* disease must continue to be carefully monitored through surveillance systems with a broad focus. In addition, the continually increasing reporting of invasive disease caused by NTHi, particularly among neonates, highlights the potential benefit of the development of a vaccine against NTHi.

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Zika Virus RNA Replication and Persistence in Brain and Placental Tissue

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Zika virus is causally linked with congenital microcephaly and may be associated with pregnancy loss. However, the mechanisms of Zika virus intrauterine transmission, replication, and tropism and persistence in tissues are poorly understood. We tested tissues from 52 case-patients: 8 infants with microcephaly who died and 44 women suspected of being infected with Zika virus during pregnancy. By reverse transcription PCR, tissues from 32 (62%) case-patients (brains from 8 infants with microcephaly and placental/fetal tissues from 24 women) were positive for Zika virus. In situ hybridization localized replicative Zika virus RNA in brains of 7 infants and in placentas of 9 women who had pregnancy losses during the first or second trimester. These findings demonstrate that Zika virus replicates and persists in fetal brains and placentas, providing direct evidence of its association with microcephaly. Tissue-based reverse transcription PCR extends the time frame of Zika virus detection in congenital and pregnancy-associated infections.

Zika virus has recently caused global concern because of an unprecedented outbreak of infection in Brazil and its association with congenital microcephaly and other adverse pregnancy outcomes, including pregnancy loss (*1–4*). Vertical transmission of Zika virus from infected mothers to fetuses has been reported (*5–7*). However, the mechanism of intrauterine transmission of Zika virus, cellular targets of viral replication, and the pathogenesis that leads to microcephaly and other congenital malformations have not yet been completely elucidated.

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Recent in vitro studies that used brain organoids, neurospheres, and human pluripotent stem cell–derived brain cells have demonstrated Zika virus infection of human neural stem and progenitor cells and have also shown that placental macrophages are permissive to Zika virus infection (*8–13*). Several studies that used mouse models have revealed that Zika virus infection of mice during early pregnancy results in infection of placenta and fetal brain, causing intrauterine growth restrictions, spontaneous abortions, and fetal demise (*14–16*). Animal models and in vitro studies, although providing valuable insights, might not exactly reflect Zika virus disease processes in humans (*9*,*17*). We previously detected Zika virus antigens in placentas of women and in human fetal or neonatal brains (*18*,*19*). However, the presence of antigens does not necessarily indicate virus replication. Previous case studies have detected Zika virus RNA by reverse transcription PCR (RT-PCR) in fetal or neonatal brains, in amniotic fluid, and in placentas of women who had acquired Zika virus infection during early pregnancy (*5*,*20–22*). Nevertheless, localization of replicating Zika virus RNA directly in the tissues of patients with congenital and pregnancy-associated infections is critical for identifying cellular targets of Zika virus infection and virus persistence in various tissues and for further investigating the mechanism of Zika virus intrauterine transmission.

Furthermore, laboratory diagnosis of congenital and pregnancy-associated Zika virus infections, particularly those involving adverse pregnancy outcomes, is also challenging because of the typically short duration of viremia (*23*,*24*). Generally, Zika virus RT-PCR can detect viral RNA in serum within 3–10 days of symptom onset (*24*,*25*). Thus, diagnosis by serum RT-PCR can be difficult for neonates who acquire Zika virus infection in utero and for women who acquire (undiagnosed) Zika virus infection during early pregnancy and later experience adverse pregnancy or birth outcomes, because Zika virus RNA generally clears from maternal/infant serum by the time the infant is born or infection is suspected. Serologic

testing by ELISA, along with plaque-reduction neutralization testing, can be useful for these cases but may not always provide conclusive Zika virus diagnosis for patients with previous flavivirus exposure or immunization (*23*–*25*) and cannot characterize the virus strain and genotype. As a part of the ongoing Zika virus public health response, we developed Zika virus RT-PCR and in situ hybridization (ISH) assays for the detection and localization of Zika virus RNA in formalin-fixed, paraffin-embedded (FFPE) tissues and tested various tissues from infants with microcephaly who died. We also tested placental/fetal tissues from a series of women suspected of being infected with Zika virus during various stages of pregnancy.

Methods

Clinical Specimens

As part of an ongoing public health response effort, we tested FFPE tissue specimens from 52 case-patients (8 infants with microcephaly who died and 44 women) for whom Zika virus infection was clinically and epidemiologically suspected. The tissue specimens were submitted during December 2015–July 2016 to the Centers for Disease Control and Prevention (CDC), National Center for Emerging and Zoonotic Infectious Diseases, Division of High-Consequence Pathogens and Pathology, Infectious Diseases Pathology Branch (Atlanta, GA, USA), by local and state health departments and pathologists for diagnostic consultation. In this series, the definition of a case-patient was 1) a pregnant woman with possible Zika virus disease (based on ≥1 of the following symptoms: fever, rash, arthralgia, or conjunctivitis and a history of residing in or traveling to countries with active Zika virus mosquito-borne transmission) or 2) an infant with clinical and epidemiologic evidence of possible Zika virus–associated congenital microcephaly. Case-patients were from the United States (n = 38, including 6 from US territories), Brazil (n = 7), and Colombia (n = 7). Tested specimens were placenta, umbilical cord, fetal tissues (from pregnancy loss) from 44 women suspected of being infected with Zika virus during pregnancy, and different portions of the brain (including cerebral cortex, pons, medulla), kidney, liver, spleen, lung, and heart from 8 infants with microcephaly who died. We also included in this analysis all available clinical, demographic, and travel history information and other relevant laboratory results from the state and local health departments and the CDC National Center for Emerging and Zoonotic Infectious Diseases, Division of Vector-borne Diseases, Arboviral Diseases Branch (including available serology and serum RT-PCR results). Clinical status of the infant, including determination of microcephaly or apparently healthy status, was based on the information (generally including anthropometric measurements, physical examination, hearing test and imaging findings) provided to CDC by the state and local health departments and referring clinicians or pathologists as of date of testing. All samples and associated medical and autopsy records were provided in the context of diagnostic consultation, a routine public health service provided by CDC. As such, institutional review was not required for the testing described in this article. Clinical and pathologic findings for 5 cases from Brazil have been previously described (*18*,*19*).

RNA Extraction, RT-PCR, and Sequencing

We designed 2 sets of primers that target the nonstructural 5 (NS5) and envelope (E) genes of Zika virus and developed RT-PCR assays for the detection of Zika virus RNA from FFPE tissues. We validated the RT-PCR assays by using various positive and negative controls. Positive controls were RNA extracted from FFPE blocks of cultured cells infected with Zika virus prototype (MR766, 1947) and Brazil 2015 strains. Negative controls were RNA extracted from FFPE cell culture controls or tissue specimens from persons with previously confirmed infection with the following viruses: dengue types 1–4, West Nile, yellow fever, Japanese encephalitis, St. Louis encephalitis, eastern and western equine encephalitis, chikungunya, herpes, parvovirus B19, cytomegalovirus, adenovirus, enterovirus, rubella, Powassan, and Lacrosse. We extracted RNA from FFPE tissues of all 52 case-patients (multiple FFPE tissue blocks per patient) by using an optimized extraction protocol as previously described (*26*) and tested the samples by newly developed Zika virus NS5 and E-gene RT-PCR and by RT-PCR for dengue and chikungunya viruses (*27*,*28*). RT-PCR assays were performed by using a QIAGEN OneStep RT-PCR Kit (Valencia, CA, USA) and 5 μl of RNA template, according to the manufacturer's instructions. The thermocycling conditions used for Zika virus NS5 gene RT-PCR were as follows: 1 cycle at 50°C for 30 min; 1 cycle at 95°C for 15 min; then 40 cycles of incubation at 94°C, 56°C, and 72°C for 1 min each; followed by 1 cycle of final extension at 72°C for 10 min. The primer sequences, annealing temperatures, and amplification product sizes of the RT-PCRs are summarized in Table 1. The NS5 (127-bp) and E (209-bp) gene–positive amplicons were directly sequenced on a GenomeLab GeXP Genetic Analysis System (AB SCIEX, LLC, Redwood City, CA, USA). The search for homologies to known sequences was performed by using the BLAST nucleotide database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). To evaluate the level of fragmentation and presence of PCR inhibitors, we also tested each sample by housekeeping gene 18S rRNA RT-PCR by using QuantumRNA Classic 18S Internal Standard (Life Technologies, Carlsbad, CA, USA).

To calculate the Zika virus RNA copy number in tissues of case-patients positive by conventional RT-PCR, we also

Table 1. Oligonucleotide primers used for RT-PCR assays*

RT-PCR	Primers	Sequence, 5′→3′	Gene target	Product size, bp	Annealing temperature, °C	Reference
Zika virus	Forward	AAG TAC ACA TAC CAA AAC AAA GTG GT	NS5	127	56	This study
	Reverse	TGT TAA GAG CGT AAG TGA CAA C				
Zika virus	Forward	TGC CCA ACA CAA GGT GAA GC	E	209	58	This study
	Reverse	ACT GAC AGC ATT ATC CGG TAC TC				
DENV1–4	Forward	AAG GAC TAG AGG TTA KAG GAG ACC C	3′ UTR	110	62	(*27*)
	Reverse	GGC GYT CTG TGC CTG GAW TGA TG				
CHIKV	Forward	TCACTCCCTGTTGGACTTGATAGA	PPG	126	55	(*28*)
	Reverse	TTGACGAACAGAGTTAGGAACATACC				

*CHIKV, chikungunya virus; DENV, dengue virus; E, envelope; NS, nonstructural; PPG, polyprotein gene; RT-PCR, reverse transcription PCR; UTR, untranslated region.

performed a quantitative real-time RT-PCR by using primers as described previously (*23*). The amount of human β-actin mRNA in the RNA extracted from each section was also determined and used as an internal reference for normalization. The relative copy number of Zika virus RNA was calculated by using the β-actin mRNA copy number, estimated at 1,500 copies/cell, as previously described (*29*).

ISH

Zika virus ISH was performed by using sense and antisense riboprobes that target multiple genes of Zika virus (Advanced Cell Diagnostics, Newark, CA, USA). ISH was developed and validated on Zika virus–positive culture cells and on various Zika virus–negative controls, including tissues from case-patients or cultures positive for dengue virus, West Nile virus, and chikungunya virus. Riboprobes targeting dengue virus and *dap*B gene (Advanced Cell Diagnostics) were also used as negative control probes for ISH. To localize Zika virus genomic RNA (using antisense probe) and negative-sense replicative RNA intermediates (using sense probe) in tissues, we performed ISH on FFPE brain and placental tissues positive for Zika virus by RT-PCR, as previously described (*30*). To examine pathologic changes in the tissue, we also analyzed tissue sections from all case-patients by routine histopathology techniques. To define specific cell types, we performed immunohistochemical analysis by using antibodies against neuronal nuclei (Abcam, Cambridge, MA, USA), glial fibrillary acidic protein (Agilent, Santa Clara, CA, USA), and CD163 (Leica Biosystems, Buffalo Grove, IL, USA) on serial sections of block positive for Zika virus by ISH from selected case-patients, according to previously described protocol (*19*).

Statistical Analyses

Statistical analyses were performed by using GraphPad Prism statistical software, version 6.0a (Graph Pad Software Inc., La Jolla, CA, USA). We compared demographic and clinical variables between the 2 groups by using the Fisher exact test (2-sided). We used the Mann–Whitney U test for 2-group comparisons of continuous data. Differences were considered statistically significant at $p<0.05$.

Results

Case-Patients Characteristics

We identified Zika virus RNA by RT-PCR in various tissue specimens from 32 (62%) case-patients (Table 2). Median maternal age (age of pregnant women and mothers of infants) was 27 years (range 15–39 years) for case-patients with positive Zika virus RT-PCR results and 29 years

Table 2. Symptom onset trimester, pregnancy outcomes and Zika virus tissue RT-PCR results of 52 case-patients*

Pregnancy or infant outcome	No. (%) case-patients, n = 52	Trimester of maternal symptom onset		Zika virus–positive case-patients, by tissue RT-PCR, no. (%), n = 32
		First, no. (%), n = 27	Second or third, no. (%), n = 24	
Spontaneous abortion	11 (21)	11 (41)	NA	9 (82)
Elective termination	3 (6)	3 (11)	NA	3 (100)
Intrauterine fetal demise†	3 (6)	1 (4)	2 (8)	0
Infant with microcephaly (fatal outcome)‡	8 (15)	8 (29)	NA	8 (100)
Infant with microcephaly (nonfatal outcome)§	5 (10)	3 (11)	1 (4)	4 (80)
Apparently healthy infant	22 (42)	1 (4)	21 (88)	8 (36)

*Of 32 case-patients with positive Zika virus tissue RT-PCR results, maternal serology (IgM and plaque-reduction neutralization test) results were consistent with recent flavivirus infection (9 case-patients) and consistent with recent Zika virus infection (4 case-patients). Zika virus infection was confirmed for 5 patients by RT-PCR at a state laboratory. For the remaining 14 case-patients, no maternal testing was performed. Of 20 case-patients with negative Zika virus tissue RT-PCR results, maternal serology (IgM and plaque-reduction neutralization test) results were consistent with recent flavivirus infection for 12 case-patients (11 who had live-born, apparently healthy, infants, and 1 who had intrauterine fetal demise). For 2 case-patients, maternal serology results were negative for Zika virus IgM, and for 7 case-patients, no maternal serologic testing was performed. Zika virus infection was confirmed for 1 case-patient by RT-PCR at a state laboratory. RT-PCR, reverse transcription PCR.
†Including 1 with microcephaly.
‡Died postnatally.
§Information about timing of symptom onset was unavailable for 1 case-patient with Zika virus–positive tissue RT-PCR results. Nonfatal, according to the information received from the case submitters as of the date of testing.

(18–43 years) for case-patients with negative Zika virus RT-PCR results; all case-patients had Zika virus infection–like symptoms. For the 32 case-patients with positive Zika virus RT-PCR results, the commonly reported signs were rash (94%), fever (59%), arthralgia (28%), headache (19%), and conjunctivitis (13%). For the 20 case-patients with negative Zika virus RT-PCR results, the most commonly reported signs were rash (80%), fever (45%), arthralgia (40%), headache (20%), and conjunctivitis (20%). No distinctive maternal clinical features between case-patients positive and negative for Zika virus by tissue RT-PCR were identified. Maternal symptom onset occurred during the first trimester for 27 (52%) case-patients and during the second or third trimester of pregnancy for 24 (46%) (Table 2). Information about timing of symptom onset was not available for 1 case-patient. Of 52 case-patients, 30 (58%) had an adverse pregnancy or birth outcome and 22 (42%) had live-born, apparently healthy, infants (Table 2).

RT-PCR and Sequencing

Brain and placental tissues from 32 (62%) of the 52 case-patients were positive by both Zika virus RT-PCR assays (gene targets NS5 and E). Sequence analysis of all positive amplicons showed 99%–100% nt identities with Zika virus Asian genotype strains currently (2015–2016) circulating in Brazil. RT-PCR results were positive for 24 (75%) of 32 case-patients with adverse pregnancy (n = 12) or birth (n = 12) outcomes and positive for 8 (36%) of 22 case-patients with live-born, apparently healthy, infants (p = 0.0082). Of 24 case-patients with positive RT-PCR results and adverse pregnancy/birth outcomes, 23 had maternal symptom onset during the first trimester, whereas all 8 case-patients with apparently healthy infants and positive RT-PCR results had symptom onset in the third trimester (p<0.0001).

Of the 13 microcephaly-associated case-patients (Table 3), 8 were infants with microcephaly and fatal outcome (died within few minutes to 2 months after birth) and 5

Table 3. Characteristics and laboratory findings for 13 microcephaly-associated case-patients*

Case-patient no.	Maternal travel history or residence	Maternal symptom onset, gestation wk/trimester	Outcome	End of pregnancy, gestational age, wk/trimester	Results of Zika virus testing performed on FFPE tissues	
					RT-PCR	ISH†
54	Brazil	4/first	Infant with microcephaly died 6 h after birth	38/third	Positive (brain); negative (placenta, spleen, kidney, lung, liver)	Positive (brain)
66	Colombia	8/first	Infant with microcephaly died 2 d after birth	26/third	Positive (brain, placenta); negative (liver)	Positive (brain)
67	Colombia	8/first	Infant with microcephaly died shortly after birth	27/third	Positive (brain, placenta); negative (liver)	Positive (brain)
68	Colombia	10/first	Infant with microcephaly died shortly after birth	27/third	Positive (brain, placenta); negative (liver)	Positive (brain), negative (placenta)
55	Brazil	NA/first	Infant with microcephaly died few min after birth	29/third	Positive (brain); negative (placenta)	Positive (brain)
53	Brazil	NA/first	Infant with microcephaly died 20 h after birth	36/third	Positive (brain); negative (placenta, spleen, kidney, heart)	Positive (brain)
37	Brazil	NA/first	Infant with microcephaly died 60 d after birth	38/third	Positive (brain)	Positive (brain)
65	Colombia	NA/first	Infant with microcephaly died few minutes after birth	28/third	Positive (brain, placenta); negative (liver)	Negative (brain, placenta)
49	Brazil, delivered in USA	7/first	Infant with nonfatal microcephaly	37/third	Positive (placenta)	Tissue NA
83	Cape Verde‡	7/first	Infant with nonfatal microcephaly	36/third	Positive (placenta)	negative (placenta)
20	Marshall Islands‡	Unknown	Infant with nonfatal microcephaly	31/third	Positive (placenta)	Tissue NA
85	Honduras‡	10/first	Infant with nonfatal microcephaly	37/third	Positive (placenta, umbilical cord)	Negative (placenta)
13	Dominican Republic	18/second	Infant with nonfatal microcephaly	39/third	Negative (placenta, umbilical cord, membrane)	ND

*FFPE, formalin-fixed, paraffin-embedded; ISH, in situ hybridization; NA, not available; ND, not done; RT-PCR, reverse transcription PCR.
†ISH results include staining by sense and antisense probes.
‡Travel history.

were women who had live-born infants with microcephaly and nonfatal outcome. Of these, 12 (92%) were positive for Zika virus by tissue RT-PCR. Zika virus RNA was detected by RT-PCR in brain tissues of all 8 infants; all had maternal symptom onset during the first trimester. Other tested tissues from infants (kidney, liver, spleen, heart, and rib) were negative by RT-PCR. Of the 17 case-patients with adverse pregnancy outcomes (Table 4), Zika virus RNA was detected by RT-PCR in placentas/umbilical cord/fetal tissues of 12 (70%); all had symptom onset during the first trimester. Of 22 case-patients who had live-born, apparently healthy, infants, including 8 case-patients with positive RT-PCR results, 21 (95%) had symptom onset during the second or third trimester.

The time frame from maternal symptom onset to detection of Zika virus RNA by RT-PCR in brains was 119–238 (mean 163) days and in placentas was 15–210 (mean 81) days. Relative levels of Zika virus RNA in the infant brain tissues (Figure 1) were ≈1,200-fold higher than those in the second or third trimester or full-term placentas (brain geometric mean 651.9 [95% CI 63.91–6,650] copies/cell; second or third trimester or full-term placentas geometric mean 0.5129 [95% CI 0.1649–1.595] copies/cell). In addition, relative levels of Zika virus RNA in the first trimester placentas (13.10 [1.718–99.87] copies/cell) were 25-fold higher than those in the second or third trimester or full-term placentas.

ISH

Overall, Zika virus RNA was demonstrated by ISH in tissues of 16 (50%) of 32 case-patients with positive Zika virus RT-PCR results. Intense signals from antisense (which binds to Zika virus genomic RNA) and sense (which binds to replicative RNA intermediates) probes were observed in brain tissues (Figure 2) of 7 of 8 infants with microcephaly. Zika virus replicative RNA, detected by using sense probe, was observed in the neural cells, neurons, and degenerative glial cells within the cerebral cortex of the brain (Figure 2). Immunostaining with antineuronal nuclei and antiglial fibrillary acidic protein was also noted in the areas of ISH staining (Figure 2). Zika virus genomic and replicative RNA was also localized in placental chorionic villi (Figure 3) of 9 (75%) of 12 women with positive Zika virus RT-PCR results who had an adverse pregnancy outcome during the first or second trimester. All 9 of these women had symptom onset during the first trimester of pregnancy. Zika virus replicative RNA was predominately observed in the Hofbauer cells of the placental chorionic villi, as identified

Table 4. Characteristics and laboratory findings of case-patients with adverse pregnancy outcome*

Case no.	Maternal travel history or residence	Maternal symptom onset, gestation wk/trimester	Outcome	End of pregnancy, gestational age, wk/trimester	Results of Zika virus testing performed on FFPE tissues	
					RT-PCR	ISH†
81	Colombia	1/first	SA	6/first	Positive (placenta)	Positive (placenta)
47	Honduras‡	5/first	SA	≈8/first	Positive (placenta)	Positive (placenta)
57	Puerto Rico‡	5/first	SA	8/first	Positive (placenta)	Positive (placenta)
56	Guatemala‡	6/first	SA	11/first	Positive (placenta)	Positive (placenta)
18	American Samoa	7/first	SA	14/second	Positive (placenta, fetal tissue)	Positive (placenta, fetal tissue)
78	Colombia	7/first	SA	11/first	Positive (placenta)	Positive (placenta)
125	Brazil	8/first	SA	11/first	Positive (placenta)	Positive (placenta)
256	Brazil	8/first	SA	13/first	Positive (placenta)	Negative (placenta)
79	Colombia	NA/first	SA	9/first	Positive (placenta)	Positive (placenta)
80	Mexico‡	6/first	SA	12/first	Negative (placenta, cord)	ND
19	Dominican Republic‡	1/first	SA	10/first	Negative (placenta)	ND
45	Honduras‡	13/first	ET	19/second	Positive (placenta); negative umbilical cord, fetal brain, liver, lung)	Positive (placenta)
76	Puerto Rico	7/first	ET	9/first	Positive (placenta)	Negative (placenta)
28	Dominican Republic‡	NA/first	ET	16/second	Positive (placenta)	Negative (placenta)
97	El Salvador	NA/first	IUFD§	34/third	Negative (placenta)	ND
92	American Samoa	NA/second	IUFD	24/second	Negative (placenta, umbilical cord, membrane)	ND
556	Marshall Islands‡	31/third	IUFD	36/third	Negative (placenta, umbilical cord, membrane)	ND

*ET, elective termination; FFPE, formalin-fixed, paraffin-embedded; ISH, in situ hybridization; IUFD, intrauterine fetal demise; NA, not available; ND, not done; RT-PCR, reverse transcription PCR; SA, spontaneous abortion.
†ISH results include staining by both sense and antisense probes.
‡Travel history.
§With microcephaly.

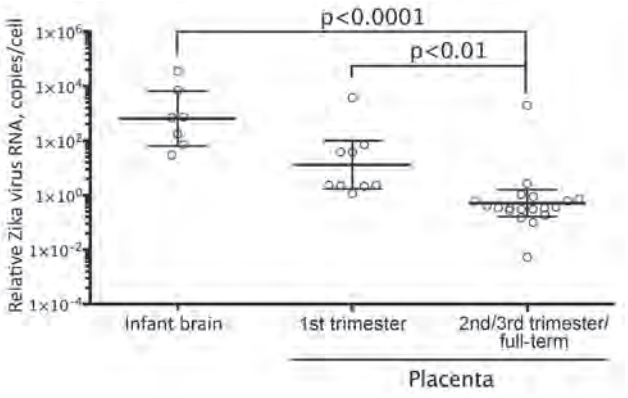

Figure 1. Zika virus RNA load levels in human brain and placental tissues. The scatter plot graph shows the relative levels of Zika virus RNA in formalin-fixed, paraffin-embedded tissue sections, which were quantified by real-time quantitative reverse transcription PCR by using primer-probe sets for Zika virus envelope gene and β-actin mRNA. β-actin mRNA was used as an internal reference gene that provided a normalization factor for the amount of RNA extracted from a section. The copy number of Zika virus RNA per cell was calculated using β-actin mRNA copy number, which was estimated to be 1,500 copies/cell. The graph shows individual data points and superimposed horizontal lines at the geometric mean, and error bars show the 95% CI for that geometric mean. p values were calculated with nonparametric 1-way analysis of variance (Kruskal-Wallis test) followed by Dunn multiple comparison tests. The relative Zika virus RNA copy numbers for second/third trimester or full-term placentas were statistically significantly lower than those for first trimester placentas or infant brain tissues.

by immunohistochemistry studies with CD163 cell marker (Figure 3). For 6 of 8 women positive for Zika virus by RT-PCR and who had apparently healthy infants, placental tissues were available for ISH testing and were all negative. Zika virus–positive culture cells and tissues from case-patients positive for Zika virus showed no signal when tested by using dengue virus and DapB probes.

Discussion

This work demonstrates evidence of Zika virus RNA replication in placentas of women who had pregnancy losses during the first or second trimester of pregnancy and in brain tissues of infants with microcephaly. We directly localized Zika virus negative-sense replicative intermediates in Hofbauer cells of placenta and neural cells and neurons by using ISH. Our findings indicate that Hofbauer cells may play a role in the dissemination or transfer of Zika virus to the fetal brain, particularly during early pregnancy. Furthermore, tissue-based RT-PCR assays described herein also extends the time frame for Zika virus detection; thus, the assays can be a valuable adjunct for the diagnosis of congenital and pregnancy-associated infections. The assays can help to expand diagnostic opportunities for Zika virus, particularly when the mother was not previously tested, the window of detection for serum RT-PCR and serology has passed, or results of Zika virus testing are inconclusive (e.g., serology consistent with recent flavivirus infection). In addition, our findings also reveal the persistence of Zika virus RNA in placenta and brain tissues, which might provide insights into the potential late or long-term sequelae of the infection.

In this series of case-patients, Zika virus clearly exhibited neurotropism. In 7 of 8 infants with microcephaly, the presence of Zika virus genomic and replicative RNA was observed in neural cells, neurons, and degenerating glial cells of cerebral cortex by ISH; whereas, all other tested tissues from these case-patients were negative by RT-PCR and ISH. Thus, our findings support those of previous in vitro and mouse studies, which demonstrated that Zika virus infects human neural stem and progenitor cells and causes severe pathologic changes in the brain but not in other visceral organs (*8–11,16*). A recent study also reported higher expression of Zika virus entry receptor AXL in radial glia (the neural stem cells of the human fetal cerebral cortex), astrocytes, and neural progenitors (*31*). Furthermore, we noted that the relative levels of Zika virus RNA in the brain tissues of infants were >1,000-fold higher than those in placentas, according to real-time quantitative RT-PCR, which also suggests replication of Zika virus in brain. Previous case reports also describe identification of Zika virus antigens and RNA predominately in fetal brain tissues by IHC and RT-PCR (*6,18–21*). We also noticed that in all microcephaly cases that were positive by tissue RT-PCR, maternal symptom onset occurred during the first trimester, which might suggest that the virus can cause abnormal brain development when infection occurs early in organogenesis. Previous studies of rubella reported that the risk for fetal infection with congenital anomalies is highest when exposure/infection occurred before 11–12 weeks of gestation and sharply decreased with increasing gestational age (*32*).

Findings also demonstrate detection of Zika virus RNA by RT-PCR in 12 (86%) of 14 women who had spontaneous abortions or fetal losses. All of these women had symptom onset during the first trimester. For 9 of these 12 case-patients (all with pregnancy losses at <19 weeks gestational age), replicative Zika virus RNA was demonstrated in Hofbauer cells (placental macrophages) of chorionic villi, suggesting direct infection and replication of Zika virus in Hofbauer cells. We have previously reported the presence of trophoblast necrosis and fibrin deposit along with viral antigens in placenta, which may also indicate placental damage by direct infection (*33*). A recent in vitro study also demonstrated that Zika virus infects and primarily replicates in Hofbauer cells (*13*). Prior studies have also identified macrophages as target cells for dissemination of dengue virus (*34*). Taken together, these findings suggest that Hofbauer cells, which

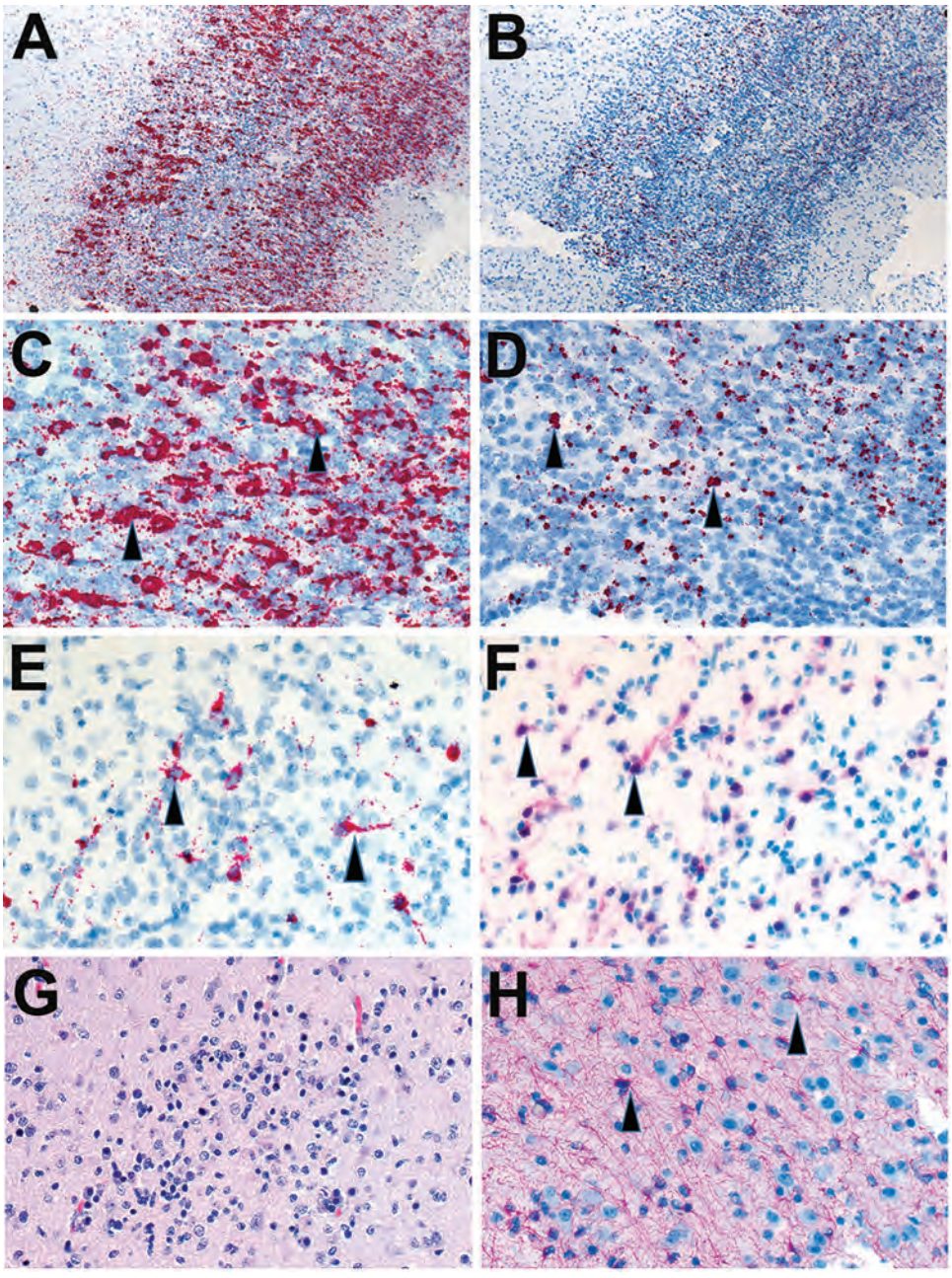

Figure 2. Localization of Zika virus RNA by in situ hybridization in brain tissues from infants with microcephaly. A) ISH with use of antisense probe. Zika virus genomic RNA (red stain) in cerebral cortex of an infant (case-patient no. 66, gestational age 26 wk). Original magnification ×10. B) ISH with use of sense probe. Serial section showing negative-strand replicative RNA intermediates (red stain) in the same areas shown in panel A. Original magnification ×10. C) ISH with use of antisense probe. Higher magnification of panel A, showing cytoplasmic staining of neural (arrowheads) and glial cells. Original magnification ×20. D) ISH with use of sense probe. Higher magnification of panel B, showing cytoplasmic staining of neural and glial cells (arrowheads). Original magnification ×20. E) ISH with use of antisense probe. Localization of negative-strand replicative RNA intermediates in neural cells or neurons (red, arrowheads) of another infant w ith fatal outcome (case-patient no. 67, gestational age 27 wk). Original magnification ×40. F) Immunostaining of neurons (arrowheads) with use of antibodies against neuronal nuclei in a serial section. Original magnification ×40. G) Hematoxylin and eosin stain showing cortical neural cells in a serial section. Original magnification ×40. H) Immunostaining of glial cells (arrowheads) with use of glial fibrillary acidic protein antibody in the same case. Original magnification ×40. ISH, in situ hybridization.

have access to fetal blood vessels, may facilitate transfer or dissemination of the virus to the fetal brain. Of note, in this case series, Zika virus RNA was not detected in any of the 3 women who had intrauterine fetal demise. Conversely, we detected Zika virus RNA by RT-PCR in placentas of women who had live-born infants with nonfatal microcephaly; however, viral load in the placentas was low. This finding may indicate temporal persistence of Zika virus RNA in placental tissues. Persistence of other viruses in immune-privileged organs (e.g., eyes, placenta, fetal brain) has been reported (*35*,*36*). Previous studies related to other arboviruses, including West Nile and chikungunya viruses, have also reported persistence of arbovirus RNA in various tissues according to RT-PCR (*37*–*39*).

Zika virus RNA (low copy number) was also detected in placental tissues of 8 women who had apparently healthy infants. In each of these women, symptom onset began during the third trimester; for 5 of them, serologic evidence of Zika virus or unspecified flavivirus infection was found. However, in these apparently healthy infants, serum/cord blood RT-PCR or serology results were negative for Zika virus or flavivirus. Detection of Zika virus RNA in the placenta by RT-PCR cannot distinguish between maternal and fetal infection. The absence of apparent abnormalities in

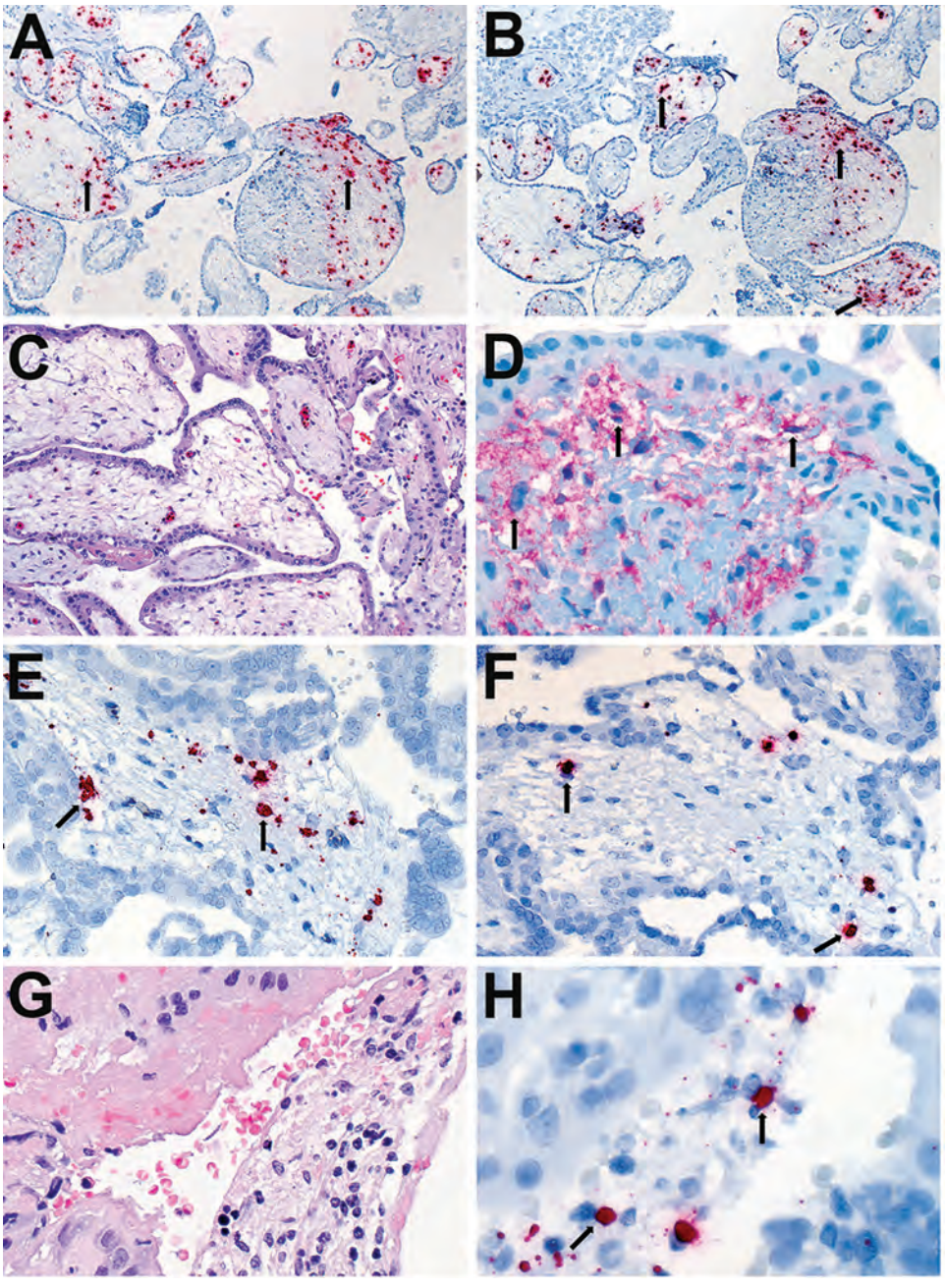

Figure 3. Localization of Zika virus RNA by ISH in placental tissues of women after spontaneous abortion. A) ISH with use of antisense probe. Zika virus genomic RNA localization in placental chorionic villi, predominantly within Hofbauer cells (red stain, arrows), of a case-patient who experienced spontaneous abortion at 11 wk gestation (case-patient no. 56). Original magnification ×10). B) ISH with use of sense probe. Serial section showing negative–strand replicative RNA intermediates (red stain, arrows) in the same cells shown in panel A. Original magnification ×10. C) Hematoxylin and eosin stain of placental tissue of a case-patient who experienced spontaneous abortion at 8 wk gestation (case-patient no. 47). Original magnification ×20. D) Immunostaining for CD163 highlighting villous Hofbauer cells in a serial section as seen in panel C. Original magnification ×63. E) ISH with use of antisense probe. Zika virus genomic RNA as seen in a serial section from the same case-patient as in panel C, showing staining within Hofbauer cells (red stain, arrows) of placental chorionic villi. Original magnification ×40. F) ISH with use of sense probe. Serial section showing negative-strand replicative RNA intermediates (red stain, arrows) in the same cells as shown in panel E. Original magnification ×40. G) Hematoxylin and eosin stain from the same case-patient as in panel C, showing inflammatory cell infiltrates in maternal side of placenta. Original magnification ×63. H) ISH with use of sense probe. Negative-strand replicative RNA intermediates (red stain, arrows) in inflammatory cells in a serial section. Original magnification ×63. ISH, in situ hybridization.

these infants could be because 1) Zika virus may not have transferred from the mother to the fetus in utero because the late-pregnancy placenta might have protected the fetus, or an effective fetal immune response might be present; or 2) the critical period of organogenesis was complete before maternal or possibly fetal infection; therefore, no apparent/major malformations were identified at the time of birth. Clinical implications for an infant with Zika virus RNA detected in the placenta, in the absence of laboratory evidence of Zika virus in the infant, are unknown. However, the negative Zika virus testing results (cord blood or serum RT-PCR and serology) in these infants should be interpreted with caution because the results could be negative because the window of Zika virus detection might have passed. Periodic monitoring of these infants may be helpful for early recognition of potential late sequelae of

congenital infections. Two recent studies observed neurologic abnormalities in infants whose mothers acquired Zika virus infection in the third trimester of pregnancy; for 1 of these infants, neurologic abnormalities were first identified at 6 months of age (*7*,*40*).

In conclusion, our findings further support the linkage of Zika virus with microcephaly, suggest its association with adverse pregnancy outcomes, and demonstrate evidence of Zika virus replication and persistence in fetal brain and placenta. This article highlights the value of tissue analysis to expand opportunities to diagnose Zika virus congenital and pregnancy-associated infections and to enhance the understanding of mechanism of Zika virus intrauterine transmission and pathogenesis. In addition, the tissue-based RT-PCRs extend the time frame for Zika virus detection and particularly help to establish a diagnosis retrospectively, enabling pregnant women and their healthcare providers to identify the cause of severe microcephaly or fetal loss.

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Spatiotemporal Fluctuations and Triggers of Ebola Virus Spillover

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Because the natural reservoir of Ebola virus remains unclear and disease outbreaks in humans have occurred only sporadically over a large region, forecasting when and where Ebola spillovers are most likely to occur constitutes a continuing and urgent public health challenge. We developed a statistical modeling approach that associates 37 human or great ape Ebola spillovers since 1982 with spatiotemporally dynamic covariates including vegetative cover, human population size, and absolute and relative rainfall over 3 decades across sub-Saharan Africa. Our model (area under the curve 0.80 on test data) shows that spillover intensity is highest during transitions between wet and dry seasons; overall, high seasonal intensity occurs over much of tropical Africa; and spillover intensity is greatest at high (>1,000/km^2) and very low (<100/km^2) human population densities compared with intermediate levels. These results suggest strong seasonality in Ebola spillover from wild reservoirs and indicate particular times and regions for targeted surveillance.

Emerging infectious diseases, a persistent threat to global public health, are often linked to rapid environmental change and increasing human mobility (*1*,*2*). Notable for its unprecedented size and geographic extent, the 2013–2015 West Africa Ebola epidemic was also the first major human Ebola outbreak outside central Africa and underscored the need for improved methods to forecast emergence in novel regions. Because the natural reservoir of the Ebola virus has not been identified (*3*) and spillovers present an irregular pattern (*4*,*5*), it remains unclear how the probability of Ebola virus disease (EVD) in human populations varies in space and time. Particularly, whether EVD follows a seasonal pattern (*6*,*7*) and which historically unaffected geographic regions may also be at risk for EVD outbreaks (*8*) are 2 important questions that remain largely unanswered. Likewise, how expanding human activities, changing settlement patterns, and increasing population density affect the probability of spillovers remains poorly resolved. Despite the absence of an obvious explanation for the timing and location of past EVD outbreaks, a set of associated social and environmental conditions that anticipate viral spillover may be broadly identifiable. Identifying the environmental correlates that bring us closer to forecasting when and where EVD risk is elevated is critical for improving surveillance and rapid response to future spillovers.

Research on Ebola during the past 2 decades has investigated spatiotemporal disease probability by using conventional time series analysis (*9*) and geostatistical models (*10*). By using time series of satellite imagery, multiple studies have suggested that Ebola spillover to humans is more likely to occur at the onset of the dry season (*7*,*11*,*12*). Noting that this pattern is not universal, Lash et al. (*13*) analyzed patterns in the time series of vegetation greenness and land surface moisture (by using a normalized difference vegetation index) for 5 spillover events and found anomalies (i.e., extreme climatic fluctuations) at a temporal scale of 20 days preceding this subset of spillover events. More recently, species distribution models have been used to map the potential geographic extent of disease probability, as in the work of Pigott et al. (*4*), who used these models to identify spatial covariates that associate with the occurrence of Ebola virus infection in humans, primates, and bats.

Despite these advances, notable technology gaps remain. For example, we know that spatiotemporal variation and seasonality are key characteristics of EVD regions, but we lack integrative models that reliably incorporate spatiotemporally varying indicators of interannual and intraannual fluctuations into the calculation of spillover probability. Further, although socioeconomic factors are believed to be important drivers of spillover for numerous zoonotic diseases, including Ebola (*14*,*15*), the relationship between human population growth and the increasing frequency of EVD outbreaks since the early 1990s remains largely unexplored. We also know that the biology of this region is strongly influenced by climatic seasonality (e.g., the timing of fruit and forage availability and animal migrations). Although such seasonality is widely suspected to affect viral amplification and transmission from wild reservoirs, time-series of climate or vegetation have not been investigated across the region of documented EVD events. In this study, we

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combine spatial data on changing human population density and distribution during the past 4 decades, satellite-derived estimates of monthly rainfall for most of the same period, and summary measures of climate in a statistical model that dynamically captures the timing of past EVD events. Our model predicts human EVD outbreaks with an estimated accuracy of 80% and shows how EVD risk shifts seasonally as a function of environmental triggers and has varied over the last 3 decades because of increases in human population and changing settlement patterns.

Methods

Ebola Spillover Origin Points and Dates

We compiled a table of all known Ebola epizootics and human outbreaks from primary sources and filtered the entries to isolate primary dates and precise locations of distinct spillover events. For human Ebola spillovers, we began with chronological lists compiled by the World Health Organization and the Centers for Disease Control and Prevention. Key sources were Lahm et al. (*16*) and Leroy et al. (*17*), who compiled reports of wildlife mortality in Gabon and the Democratic Republic of the Congo; reports by ethnologists observing great ape populations in other regions; coordinates of locations from Mylne et al. (*18*); and locations and associated information from Kuhn's compendium (*5*).

To divide incident reports into discrete spillover events, we separated primary spillovers from secondary occurrences on the basis of widely accepted chronological, geographic, or genetic distances. For example, where viral sequence data indicated that multiple spillover events had occurred, we considered them as such even if they overlapped spatially or temporally. Most events were reported as points. When events were reported as polygons (3 cases), we used polygon centroids as point locations. In contrast to Pigott et al. (*4*), we excluded data derived from sampling of healthy bats not associated with a spillover event. Because we were seeking to identify potential climatologic triggers, the timing of the spillover was taken to be the earliest report (often unconfirmed) of either human or animal disease rather than the first date of confirmed infection in either humans or animals. Following this procedure, a primary list of 66 spatiotemporal candidate spillover points was reduced to a final list of 44 spillover events (Figure 1; online Technical Appendix 1, https://wwwnc.cdc.gov/EID/article/23/3/16-0101-Techapp1.xlsx).

Spatial Predictors

To exclude arid and semi-arid regions, which are unlikely to harbor potential Ebola reservoir species and differ sharply in climate from locations where human EVD has occurred, we defined the region of interest as the portion of Africa receiving >500 mm rainfall annually. For this region, we assembled spatial data that capture the major sources of variation in climate and landcover. Following Pigott et al. (*4*), we chose an enhanced vegetation index (*19*) and potential evapotranspiration (*20*) to represent composite axes of coarse environmental variation.

Candidate Triggers

To characterize spatiotemporal variation at seasonal, interannual, and decadal scales, we compiled 3 datasets. First,

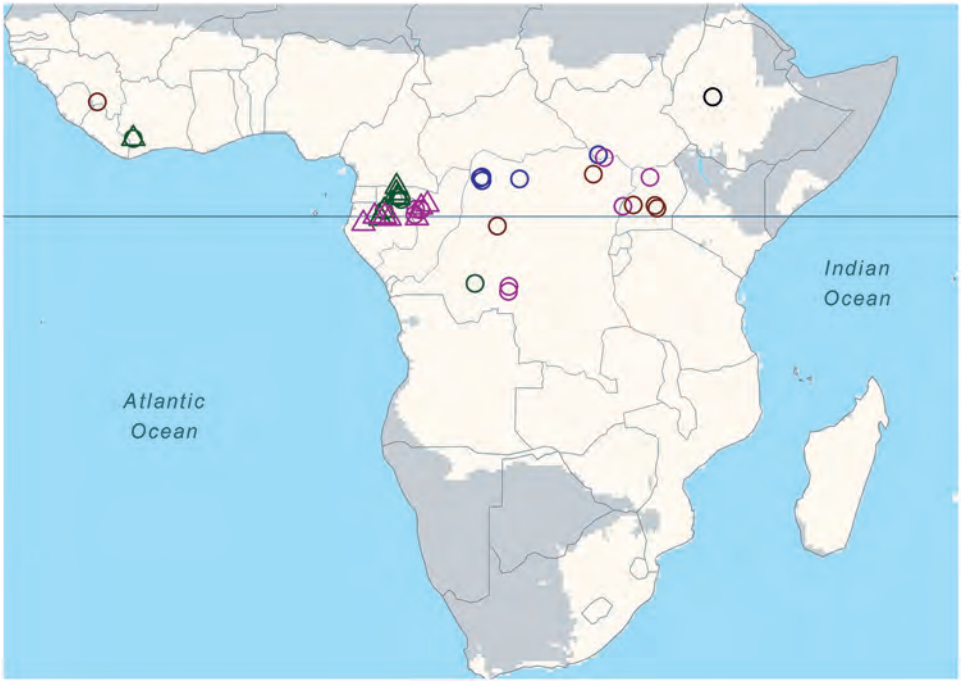

Figure 1. Locations of known Ebola virus spillover events, Africa, 1960–2010. Light-shaded area indicates the focal region in Africa of annual rainfall >500 mm. Open circles indicate human spillovers, open triangles infection/mortality in nonhuman primates or in other mammals. Yellow, blue, green, magenta, and black indicate the 5 respective decades during 1960–2010. Solid horizontal line marks the equator. No known Ebola spillovers occurred in the 1980s.

we compiled population count grids for Africa for 1960, 1970, 1980, 2000, 2005, 2010, and 2015 at 2.5 arc-minutes scale (≈25 km^2 at the equator) from the Gridded Population of the World version 3, produced by the Columbia University Center for International Earth Science Information Network (http://sedac.ciesin.columbia.edu/data/collection/gpw-v3). After linearly interpolating counts by grid cell for intervening years, we $\log_{10}$-transformed values of human population and created 3 population bins according to $x \leq 10^2$, $10^2 < x < 10^3$, and $x \geq 10^3$.

Second, we aggregated monthly rainfall from daily rainfall estimates obtained from the Rainfall Estimator (*21*). This data product was developed in 1998 by the Climate Prediction Center at the National Oceanic and Atmospheric Administration and is available at high (0.1°) spatial resolution for January 1983 to the present.

Third, in addition to actual monthly rainfall, we created a rainfall anomaly index as a means of incorporating the potential importance of relative rainfall. For the time series of 384 monthly rainfall rasters, we divided the value of each month-location by the maximum value for that location to create a set of 384 scaled raster images corresponding to the original monthly rainfall raster images.

Model Fitting and Validation

We restricted analysis to the 37 (80.5%) of 44 EVD events occurring since 1982, the period for which monthly rainfall estimates across Africa were available. We sampled 100,000 random background points from within the portion of Africa receiving >500 mm rainfall annually and randomly assigned each to 1 of 384 months during 1983–2014. Spillover occurrence points and background points were divided into 2/3 training and 1/3 test sets. Because actual monthly rainfall at month and site of EVD outbreak varied considerably, we stratified by rainfall, first ranking points by rainfall amount and then assigning every third point to the test set.

Model Testing

We modeled Ebola spillover intensity, the average density or expected number of points per unit area and time, using bagged logistic regression models with main effects only (*22*). Bagging (bootstrap aggregating) is a machine learning approach that uses the predictive power generated from ensembles of models based on small subsets of the data (*23*). By using all 5 predictors described, we fit 1,000 models in which we randomly sampled 10 of the 22 outbreaks in the training dataset and 100 of 100,000 training background points. We predicted each of the 1,000 fitted models on both the training and test datasets. Taking the mean of predicted spillover intensity across model iterations, we compared average predicted spillover intensity for training and test points with labels at each point (known EVD event vs. otherwise) in each dataset to gauge the accuracy of the models.

Risk Mapping

The set of known EVD events represent a spatiotemporal point process. Point processes are described by an intensity function (i.e., the average density or expected number of points per unit area and time). Therefore, after validation, we used the complete dataset (37 spillover and 100,000 background points) to retroactively predict Ebola spillover intensity across the entire portion of Africa receiving >500 mm rainfall annually for all 384 months for which gridded rainfall data were available (January 1983–December 2014) using human population estimates for 2015. We then averaged the resulting 384 monthly rasters to map seasonal shifts in predicted Ebola spillover intensity across Africa. To map the change in spillover intensity as a function of changes in human population size and distribution across 4 decades, we averaged predicted intensity across all months of 1975 and 2015, then took the difference between annual spillover intensity in 2015 and annual spillover intensity in 1975 across the region of Africa receiving >500 mm rainfall annually.

Detailed methods are provided in online Technical Appendix 2 (https://wwwnc.cdc.gov/EID/article/23/3/16-0101-Techapp2.pdf), and the R code used is provided in online Technical Appendix 3 (https://wwwnc.cdc.gov/EID/article/23/3/16-0101-Techapp3.pdf). All data and code are available online (https://figshare.com/articles/ebola_spillover_intensity_final_Rmd/4234280).

Results

Predictive accuracy of the bagged model of EVD intensity trained on the 2/3 training dataset was high. Area under the receiver-operator curve was 0.83 when evaluated on the training dataset and 0.80 when evaluated on 1/3 of the data that were withheld from model training. Overall accuracy ([true positives + true negatives] / total points) was 53% for prediction on the test set. Mean annual Ebola spillover intensity was highest where the enhanced vegetation index is highest in the wettest portions of tropical Africa. For locations within the humid tropics of Africa, predicted spillover intensity was generally, but not always, lowest in dry months (rainfall <50 mm) (Figure 2; online video, https://wwwnc.cdc.gov/EID/article/23/3/16-0101-V1.htm). Across sites, modeled spillover intensity in months of intermediate rainfall (100–250 mm) was equal to or exceeded that in high (>250 mm) rainfall months (Figure 3). Whereas central Africa exhibits relatively constant spillover intensity throughout the year (particularly within the narrow equatorial region of 15°–30° longitude), we found spillover intensity to be highly seasonal in southern Africa and somewhat variable in West Africa. These results extend the proposed potential range of spillover far beyond the locations of past outbreaks. Compared with previously published spatial models of spillover that did not include temporally varying predictors, our results suggest that a

much larger area of Africa is at moderate to high risk for spillover during some months of the year, including much of East Africa, Madagascar, and south central Africa (e.g., Angola and Zambia) and a large portion of West Africa (online video). Although Ebola spillover intensity in seasonally at-risk regions peripheral to central Africa is much lower than in high-intensity central Africa itself, predicted spillover intensity at sites in Angola, Mozambique, and Ethiopia is comparable to that predicted at known spillover locations in South Sudan and Gabon. Predicted spillover intensity over a large portion of Madagascar is similar to that of central Africa (Figure 3). Thus, within the African tropics, the potential for Ebola spillover appears to be geographically widespread.

The effect of human population on Ebola spillover intensity is much smaller than climatic or seasonal effects. The change in average annual spillover intensity did not change markedly for much of Africa as population increased during 1975–2015 (Figure 4), whereas spillover intensity exhibited striking shifts with climate and seasonality (Video). Nevertheless, our model does show that spillover intensity differs by human population density. Mean annual spillover intensity was lowest where population size per 25 km^2 grid cell was intermediate ($10^2<x<10^3$) and highest where population density was low ($x\leq100$) (online Technical Appendix 2 Figure 1). Large changes in spillover intensity (±5%) during 1975–2015 appear to result mainly from population increases. In comparing 2015 to 1975 population density, shifts from intermediate-to-high population densities have generated increased Ebola spillover intensity, particularly in West Africa and the region surrounding Lake Victoria, and shifts from low-to-medium population densities have reduced spillover risk. Similarly, settlements along transportation corridors have increased in population to intermediate densities, leading to substantial declines in predicted spillover intensity. However, as a result of population consolidation over large areas of central Africa, some remote districts have declined in population, typically increasing predicted spillover intensity (Figure 4; Figure 5).

These results quantify a spatiotemporal pattern in the risk for Ebola spillover in 2 specific ways: first, as raw estimates by the model algorithm that can be directly compared between months and across locations (Figure 3); and second, as percentile ranks of these estimates or relative

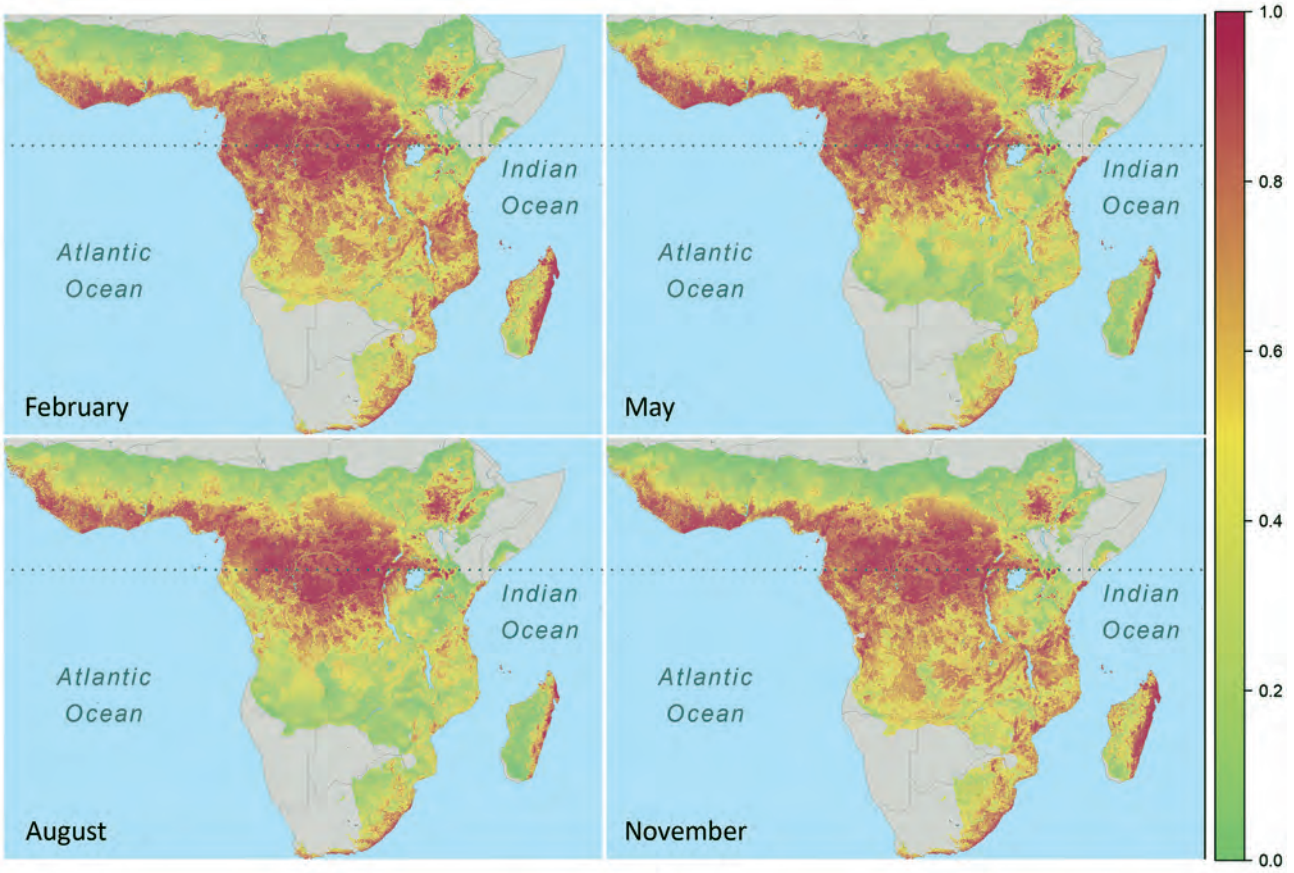

Figure 2. Seasonal spatiotemporal dynamics of Ebola virus spillover intensity (i.e., average density or expected number of points per unit area and month) as percentile values ranking predicted intensities at all grid cell locations within the region of Africa where annual rainfall was >500 mm for all months from January 1983 through December 2014. Panels capture shifts in the geographic pattern of spillover intensity seasonally. Dotted horizontal line marks the equator.

spillover intensity (online video). Percentile ranking adjusts for model miscalibration because some spillovers may not have been observed and because of the overrepresentation of spillover events in base logistic regression models. As such, percentile ranking preserves discriminability (i.e., classification accuracy as measured by area under the receiver-operator curve performance), even when probabilities are not well calibrated. Whether raw or ranked, spillover intensities are a measurement of risk, with values proportional to the probability of a spillover that changes as a function of environmental conditions based on the best information available on the location and timing of unique spillover events. Although the transmission, dynamics, and possibly the seasonality of different viral strains may differ, our approach, constrained by the small number of spillovers, properly considers EVD as a syndrome caused by all known strains of the Ebola virus. By constructing models to compare the covariates associated with this set of known spillover events to the background possibilities from which they might have been drawn, which we accurately approximate by using a sample of 100,000 random points, we have robustly determined how the intensity of Ebola spillovers changes with observable covariates.

Discussion

These results indicate that 1) there is a geographic gradient of annual Ebola spillover intensity that peaks in central Africa but extends during at least some months of the year through a large portion of tropical Africa not previously considered to be at high risk (*4*,*24*), including the tropical/subtropical forest/woodland regions of Ethiopia, Angola, Zambia, East Africa, and Madagascar; 2) there is substantial seasonal fluctuation in the spatial pattern of Ebola spillover intensity; 3) there is a temporal gradient in spillover intensity in which the driest months show the lowest intensity and intensity peaks or plateaus in months of intermediate rainfall; and 4) increases in human population density may increase Ebola spillover risk in West and central Africa. Ebola spillover intensity is greatest when regions that are typically very wet make the transition to or from dry periods. This result corroborates the finding from previous studies (*7*,*11*) linking EVD events to preceding dry-to-wet transitions through time series analysis of data from a normalized difference vegetation index. Within predominantly or seasonally wet climate zones in particular, our results show Ebola spillover intensity to be highest in moderately dry months and lowest in extremely dry months.

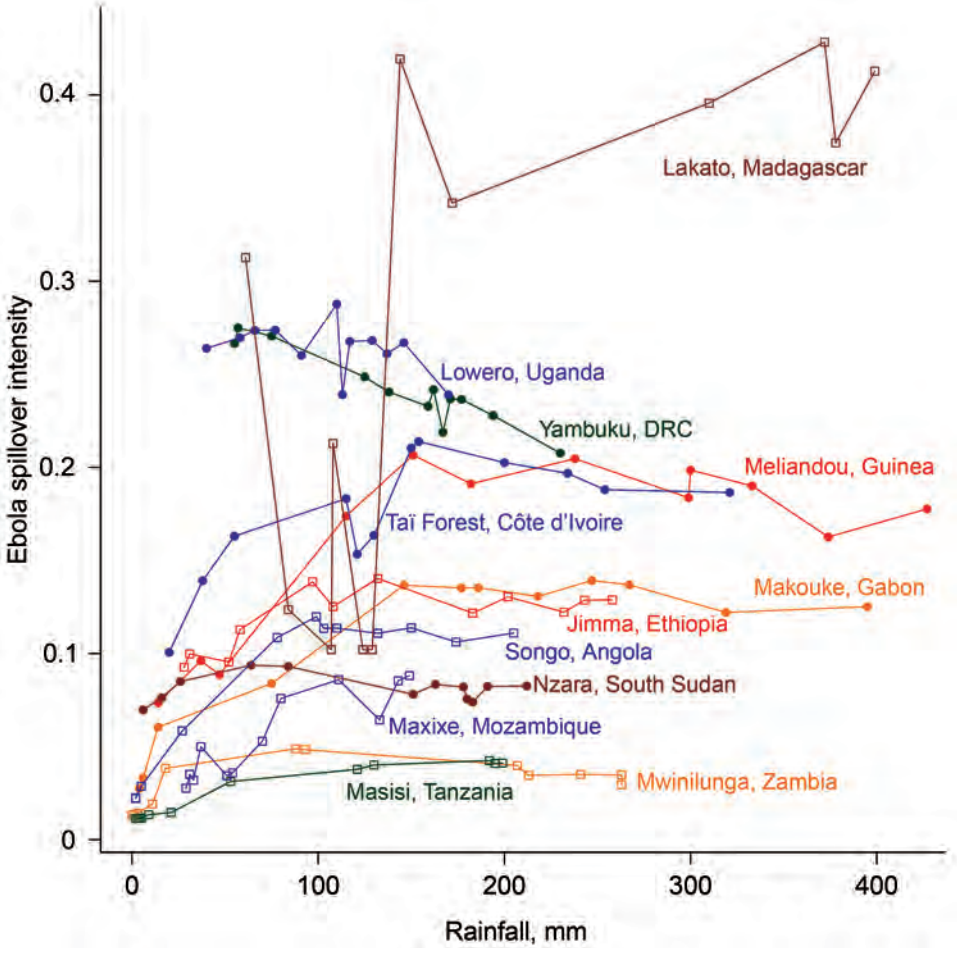

Figure 3. Phase graph showing the relationship between mean monthly rainfall and raw Ebola spillover intensity (defined as average density or expected number of points per unit area and/or time) for known Ebola virus disease locations in West and Central Africa (closed circles) and locations in northeastern or southern Africa where model results indicate moderate to high Ebola spillover intensity seasonally (open squares). Points are ordered by least to greatest monthly rainfall at each site. Dotted horizontal line marks the equator. DRC, Democratic Republic of the Congo.

Seasonal dynamics in spillover intensity are most pronounced where rainfall seasonality is greatest (i.e., outside the less seasonal and wetter rainforest biome of central Africa, where EVD events have been most frequent and spillover intensity is most steady throughout the year). Strong seasonal patterns may be related not only to seasonal drivers, such as rainfall, but to migration patterns and seasonal competence of wildlife reservoirs. Seasonal effects on resource availability may drive migrations or other changes in movement patterns that, in turn, may affect population density, social behaviors, and contact rates among hosts (*25*). Seasonal changes may also alter the frequency of host encounters with infective agents or material in the environment, and host immune defenses can shift with annual reproductive cycles (*26*). Seasonality is also likely to alter human behavior, including hunting effort, level of bushmeat consumption, or, more generally, the degree and kind of contact with wildlife.

Our model finds that Ebola spillover intensity varies temporally as a function of climate variables without explicitly incorporating sociocultural dimensions, such as land use, which was not available as a time series, or biotic features, such as the ranges of suspected reservoir hosts. Therefore, the degree of human disease intensity at locations far from documented EVD events may also depend on whether the range of a necessary reservoir also extends to these points. In recent work, species distribution models were used to predict the ranges of potential mammal reservoirs and the degree of overlap of predicted ranges with Ebola and Marburg spillovers to suggest likely mammalian reservoirs (*27*). Among the taxa that overlapped with all EVD sites were the sun squirrel genus (*Heliosciurus*) and the straw-colored fruit bat (*E. helvum*), both of which had predicted ranges covering nearly all of tropical Africa (with the exception of Madagascar, where *E. dupreanum* is present), where our models predicted high Ebola spillover intensity at least seasonally. Thus, our predictions across continental Africa may adequately reflect the biotic component of risk. However, an important next step would be to assess whether the presence of suitable animal hosts or cultural or socioeconomic factors in Madagascar and East Africa make this region a priority for surveillance.

Our model was trained by using great ape and human EVD events. Great ape spillover events (usually observations made by primatologists and wildlife researchers within reserves, in this dataset restricted to Gabon and the Democratic Republic of the Congo) are associated with low human population density, whereas our model associates

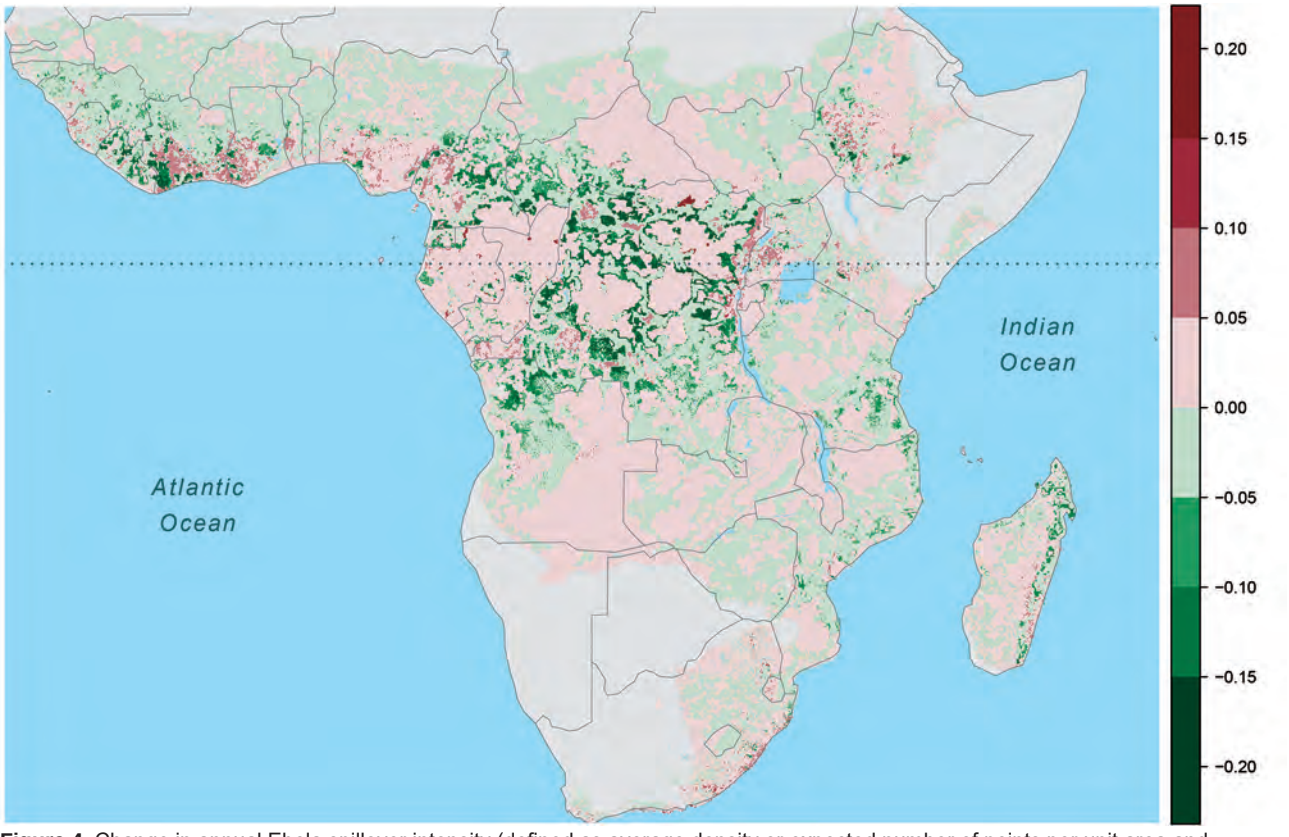

Figure 4. Change in annual Ebola spillover intensity (defined as average density or expected number of points per unit area and time), Africa, 1975–2015. Warm colors indicate increased spillover intensity; cool colors indicate decreased spillover intensity. Dotted horizontal line marks the equator.

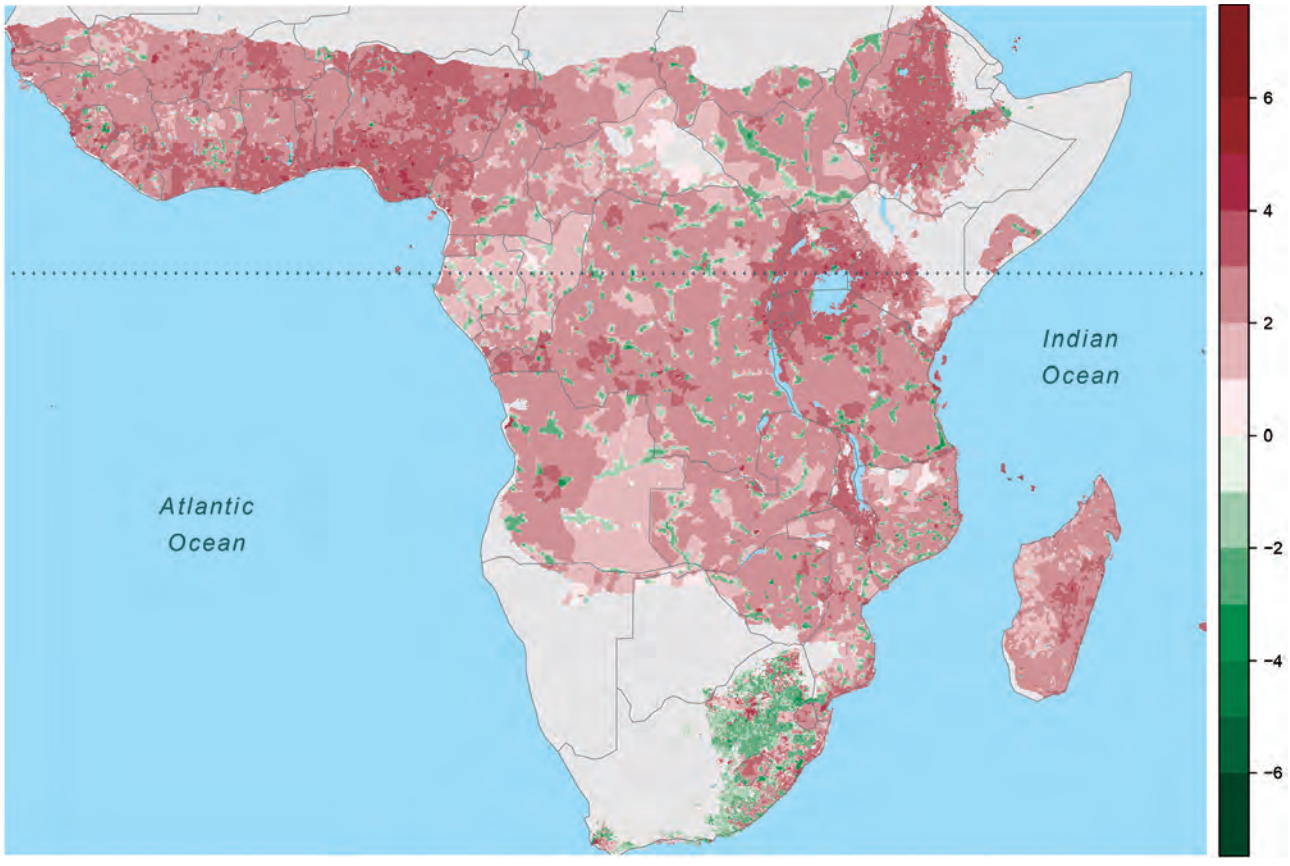

Figure 5. Change in human population size (log_{10}/25 km^2 grid cell), Africa, 1975–2015. Warm colors indicate increased population size, cool colors population declines. Dotted horizontal line marks the equator.

human spillovers with high human population densities (>10^3 persons/25 km^2). At low population densities, epidemic spread is less likely, and deaths in remote outposts may go unreported. The link between human population density and Ebola spillover intensity could be simply a function of increased reporting at high population densities (and some locations with very low population densities). Alternatively, increased contact with or consumption of wildlife as population density increases, or perhaps the increased abundance of reservoir or bridge reservoir species at either high or low human population density (or both) could drive the relationship. Substantially increased raw Ebola spillover intensity (>5%) as a result of population increases is most apparent in areas of West Africa but could eventually include central Africa if urbanization or population consolidation continues there. We note that human population was a much less important predictor than variables capturing climate and seasonality.

In conclusion, we developed a model that predicts a pattern of widespread but seasonally very dynamic Ebola spillover intensity in savannah and humid tropical regions of Africa from the set of known spatiotemporal EVD points (n = 37 since 1990) and spatially and temporally high-resolution rainfall and population data for Africa. Ebola virus, though not the strain that led to the recent outbreak, was known to be circulating in West Africa before 2014 (*28–30*). However, the potential for a major human outbreak, by far the most deadly Ebola outbreak to date, was not foreseen. Answering the need for improved forecasting, surveillance, and preparation for rapid response, our model uses the best available spatiotemporal predictors and an ensemble modeling approach to accurately identify geographic regions and seasons of elevated Ebola spillover intensity, and suggests that the socio-ecologic conditions that triggered the initial spillover in Guinea may prevail over a much larger area and at a higher temporal frequency. A key public health policy implication is that some level of Ebola surveillance should be extended to regions outside of central and West Africa. Furthermore, the spatiotemporal pattern of Ebola spillover intensity we report could be used as an early warning system to inform the design of surveillance activities.

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New *Mycobacterium tuberculosis* Complex Sublineage, Brazzaville, Congo

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Tuberculosis is a leading cause of illness and death in Congo. No data are available about the population structure and transmission dynamics of the *Mycobacterium tuberculosis* complex strains prevalent in this central Africa country. On the basis of single-nucleotide polymorphisms detected by whole-genome sequencing, we phylogenetically characterized 74 MTBC isolates from Brazzaville, the capital of Congo. The diversity of the study population was high; most strains belonged to the Euro-American lineage, which split into Latin American Mediterranean, Uganda I, Uganda II, Haarlem, X type, and a new dominant sublineage named Congo type (n = 26). Thirty strains were grouped in 5 clusters (each within 12 single-nucleotide polymorphisms), from which 23 belonged to the Congo type. High cluster rates and low genomic diversity indicate recent emergence and transmission of the Congo type, a new Euro-American sublineage of MTBC.

Despite the availability of antituberculous drugs for the past 60 years, tuberculosis (TB) remains a major health threat worldwide. In 2014, the World Health Organization registered 6 million new TB cases, and 1.5 million affected persons died of infection with *Mycobacterium tuberculosis* complex (MTBC) strains, the causative agent of TB (*1*). Congo (Republic of Congo), in Central Africa, has a population of ≈4 million inhabitants and is considered to be a high TB incidence area; incidence is 381 cases/100,000 inhabitants (*1*). Approximately one fourth of the population agglomerates in its capital city, Brazzaville.

Despite the serious situation, precise data on disease dynamics and recent transmission patterns guided by modern molecular epidemiologic tools are only sparsely available. Molecular epidemiology is useful for analyzing MTBC strain diversity and transmission dynamics in low- and high-incidence settings (*2*,*3*). Furthermore, molecular typing has shown that MTBC has a diverse population structure with manifold lineages that show large differences in geography and pathobiological properties, such as the development and spread of drug resistance (*4*,*5*).

To address current knowledge gaps, we determined the population structure of MTBC isolates from patients with pulmonary TB in Brazzaville. Samples were collected from patients at the Centre Antituberculeux de Brazzaville during February–June 2011 (*6*). We investigated the population structure and transmission patterns by a combination of classical genotyping and whole-genome sequencing (WGS). Single-nucleotide polymorphisms (SNPs) detected by WGS were used for phylogenetic lineage classification and similarity analysis estimating recent transmission rates. This approach enabled detailed insight into the population structure and phylogeny of MTBC strains circulating in Brazzaville. Moreover, we describe a new predominant sublineage, the Congo type, which most likely forms a larger transmission network in the study area.

Methods

Study Design

The patient population was reported previously (*6*). In brief, 775 consecutive patients seeking care at the Centre Antituberculeux de Brazzaville during February–June 2011 were evaluated for pulmonary TB according to the national diagnostic algorithm (*6*). The institutional ethics committee, Comité d'Ethique pour la Recherche Scientifique, in August 2010 (no. 00000067/DGRST/CERSSA) approved the study. Informed consent for study participation, as well as permission to use isolates from samples provided, were obtained from all enrolled participants.

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Samples with the highest Ziehl-Neelsen score (semiquantitatively classified in categories 1+, 2+, or 3+ in on-site laboratories based on microscopic findings) out of at least 2 positive sputum samples from 1 patient (n = 211) were shipped to the Research Center Borstel (Borstel, Germany) for culture, drug susceptibility testing, genotyping, and WGS.

Sample Processing, Culture, and Drug Susceptibility Testing

Approximately 5 mL of each specimen was homogenized by digestion for 1 min at room temperature with 1 mL of N-acetyl L-cysteine (25 mg/mL) in phosphate buffer (pH 6.8) and vortexed with several 4-mm glass beads for 30 s. A 5-mL aliquot was decontaminated by using 1% NaOH and concentrated at 4,000 × *g* for 15 min. The sediment was then reconstituted to 2.5 mL by using phosphate buffer pH 6.8 to make the inoculum for smears and cultures. Sputum was cultured by using the conventional Löwenstein-Jensen growth medium followed by determination of mycobacterial species according to standard techniques (*7*). Samples for susceptibility testing of first-line drugs were processed as described previously (*8*). Drug susceptibility testing was performed by using the BACTEC MGIT system (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Samples without antimicrobial drugs served as growth controls. Genomic DNA was extracted from sputum cultures on Löwenstein-Jensen medium, by using a standard cetyltrimethylammonium bromide–NaCl method (*3*).

Traditional Genotyping

We performed spacer oligonucleotide typing (spoligotyping) as described by Kamerbeek et al. (*9*). PCR-negative controls were included in which distilled, diethyl pyrocarbonate-treated H_2O was added instead of DNA. Genomic DNA of *M. tuberculosis* H37Rv and *M. bovis* BCG were included as controls. In addition to spoligotyping (*10*), we conducted mycobacterial interspersed repetitive unit–variable number tandem repeat (MIRU-VNTR) typing based on 24 loci as described previously (*11*); both 24-loci MIRU-VNTR typing and spoligotyping data analysis was performed by using the tools implemented at the MIRU-VNTRplus website (*12*). Genomic DNA of *M. tuberculosis* H37Rv was included as a positive control. We used the MIRU-VNTRplus nomenclature server (*11*) in addition to the definition of shared spoligotypes to assign a unique MTBC 15-9 number to each 24-loci MIRU-VNTR combination. Data were analyzed further only if all controls showed the expected outcome. All traditional genotyping techniques were performed at the Research Center Borstel, Leibniz Center for Medicine and Biosciences (Borstel, Germany).

WGS and Data Analysis

We prepared libraries for next-generation sequencing from genomic DNA by using the Nextera XT library preparation kit and run with Illumina-supplied reagent kits on the HiSeq and MiSeq systems (Illumina, San Diego, CA, USA), according to the manufacturer's recommendations. For 1 isolate (9679-00), genomic DNA was sequenced by GATC Biotech AG (Konstanz, Germany). WGS of the strains of the study population was conducted at the Research Center Borstel, Leibniz Center for Medicine and Biosciences. NGS data of sequenced isolates was submitted to the EMBL-EBI ENA sequence read archive (PRJEB9545).

We mapped sequence reads to the *M. tuberculosis* H37Rv genome (GenBank accession no. NC_000962.3) with the SARUMAN exact alignment tool (*13*). Genomic coverage was at least 50-fold for all isolates. Customized Perl scripts were used to extract SNPs from mapped reads, requiring a minimum coverage of 10 reads and a minimum allele frequency of 75% as detection thresholds (*14*). We excluded SNPs in resistance-mediating genes and repetitive regions from the phylogenetic analysis (*15*). Moreover, to avoid calling SNPs because of indel-related artifacts, we excluded SNPs within ± 12 nt from each other (*16*). Positions that matched these thresholds in all isolates were considered as valid and used for a concatenated sequence alignment.

We then calculated a pairwise distance matrix from concatenated SNP positions by Perl scripts, with +1 distance between paired isolates for each mismatching base, and plotted data in GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). On the basis of the distance matrix, we grouped isolates into putative transmission networks by incrementally accumulating all isolates with a maximum distance of 5 or 12 SNPs, respectively, to the nearest neighbor into 1 group (*16*). On the basis of the WGS data, we classified isolates into known phylogenetic groups according to the set of informative SNP positions published by Coll et al. (*17*).

We calculated the phylogenetic tree using the maximum-likelihood method and the general time reversible (GTR) substitution model, rate heterogeneity, without invariant sites using a gamma distribution as well as bootstrap resampling. Substitution models were tested and trees calculated by using MetaPiga software version 3.1 (*18*) and the maximum-likelihood ratio test (*19*). We applied midpoint rooting with FigTree software version 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/) and performed formatting by using the online tool Evolview (*20*). Specific SNPs for the Congo-type sublineage, and the more distant undefined strain 8095/11, were extracted by the ancestral states reconstruction method, implemented in the MetaPiga version 3.1 software, for both the specific and the common node. The maximum parsimony tree for visualizing genome-based clusters was calculated with Bionumerics 7.5 software (Applied Maths, Kortrijk, Belgium).

We used the concatenated sequence alignment in a Bayesian coalescent analysis with BEAST version 1.8.2 to infer node ages in the genealogic tree (*21*). A tip dating approach was not possible; therefore, we used a strict molecular clock prior of 1×10^{-7} substitutions per site per year and compared different demographic models and a GTR versus Hasegawa, Kishino and Yano substitution model using a chain length of 10 million and sampling of every 1,000 generations with a burn-in of 10% that resulted in adequate mixing of the Markov chains and effective sample sizes in the thousands. The comparison of the likelihoods of each run with Tracer version 1.5 showed very strong support of the GTR substitution model over Hasegawa, Kishino and Yano ($\log_{10}$ Bayes factors >93) and no preference for a particular demographic model; thus, we used a coalescent constant size model, representing the most straightforward approach. Resulting data were combined in a maximum clade credibility tree by using TreeAnnotator version 1.8.2 to infer node ages and highest posterior density intervals (*21*).

Results

Study Population

Results of sputum smear microscopy, radiographic abnormalities, and HIV infection were reported previously (*6*). In brief, 211 sputum samples of patients with suspected TB were sent to the National Reference Center for Mycobacteria (Borstel, Germany); 75 cultures yielded positive results. Phenotypic characterization identified 1 *M. intracellulare*, 6 *M. africanum*, and 68 *M. tuberculosis* isolates. We excluded the *M. intracellulare* isolate from further analysis. The mean age ± SD of the 74 TB patients was 33.86 ± 11.65; 69 (93%) patients were <50 years of age, and 66% were male. The patients' residences were distributed in the different districts of Brazzaville as follows: southern part, 31% from Makélékélé and 4% from Bacongo; and northern part, 22% from Talangai, 8% from Poto-poto, and 7% from Ouenzé (4%). Of the 74 study participants, 13 (18%) were HIV co-infected.

Drug Susceptibility Patterns

We determined phenotypic drug susceptibility patterns for the first-line anti-TB drugs isoniazid (INH), rifampin (RIF), ethambutol (EMB), and pyrazinamide (PZA) for all strains. In case resistance against 1 of these drugs was detected, streptomycin (STR) and second-line antimicrobial drugs were included in the analysis. Of the 74 MTBC strains, 71 (96%) were fully sensitive to all the first-line anti-TB drugs. Three (4%) isolates were resistant: 1 isolate was resistant against INH and STR, and 2 isolates exhibited a multidrug-resistant (MDR) phenotype with resistances against INH, RIF, PZA, and EMB (Table). The 2 MDR strains underwent susceptibility testing on second-line anti-TB drugs; 1 isolate was resistant to ethionamide. No extensively drug resistant strains were identified.

Population Structure of the MTBC Isolates

For all isolates, we successfully performed classical genotyping and WGS. Overall, we detected 18,059 SNP positions, which we used for further interrogations. On the basis of these analyses, we classified the *M. tuberculosis* strains into the main phylogenetic lineages Euro-American (n = 64); Delhi/Central Asian (n = 2; Coll lineage 3 and sublineage 3.1.1); Beijing (n = 1; Coll sublineage 2.1.1); East African Indian (n = 1; Coll sublineage 1.2.2); and *M. africanum* West African-1 (n = 6; Coll lineage 5). The Euro-American strains split into Latin American Mediterranean (LAM; n = 12; Coll sublineages 4.3.2, 4.3.4.1, 4.3.4.2, 4.3.4.2.1 and 4.3.3); Uganda I (n = 7; Coll sublineage 4.6.1.2); Uganda II (n = 1; Coll sublineage 4.6.1.1); Haarlem (n = 4; Coll sublineage 4.1.2.1); and X type (n = 3; Coll sublineage 4.1.1.1). Eleven Euro-American strains not well classified by MIRU-VNTR/spoligotyping (mainly T) belong to the Coll sublineages 4.1.2, 4.2.2, 4.6, 4.7, 4.8, and 4.9. Although few 4.7 strains were present in the collection analyzed by Coll et al. (*17*), they are dominant in our study collection and thus were termed Congo type (n = 26). These strains form a clear-cut branch in the MIRU/spoligotyping (data not shown) and SNP-based phylogeny (Figures 1, 2). Thus, these strains most likely represent a new Euro-American sublineage circulating in the region.

For an in-depth view on the population structure and to define the position of the Congo-type strains in the MTBC phylogeny, we analyzed the SNPs detected by WGS together with a set of reference strains (65 strains) previously used in the MIRU-VNTRplus dataset (*11*). Our reference collection comprises 3 clinical isolates of the major lineages of the MTBC and the type strains *M. tuberculosis* H37Rv ATCC 27294, *M. bovis* ATCC 19210, and *M. africanum* West African 2 ATCC 25420 (*22*). On the basis of the 18,059 SNP positions, we calculated a maximum-

Table. Description of lineage and associated *rpoB*, *katG*, and *fabG1-InhA* mutation identifiers in drug-resistant *Mycobacterium tuberculosis* isolates, Congo*

Isolate code	Resistance	Gene	Nucleotide change	Amino acid substitutions	Lineage
8032/11	INH, STR	*katG*	AGC-315-ACC	Ser-315-Thr	Congo type
8114/11	INH, RIF, STR, EMB, PZA, ETH	*katG*	AGC-315-ACC	Ser-315-Thr	Uganda I
		rpoB	GAC-516-GTC	Asp-516-Val	
		inhA	–102G/A	NA	
8125/11	INH, RIF, STR, EMB, PZA	*katG*	AGC-315-ACC	Ser-315-Thr	Beijing
		rpoB	GAC-516-TAC	Asp –516- Tyr	

*EMB, ethambutol; ETH, ethionamide; INH, isoniazid; NA, not applicable; PZA, pyrazinamide; RIF, rifampin; STR, streptomycin.

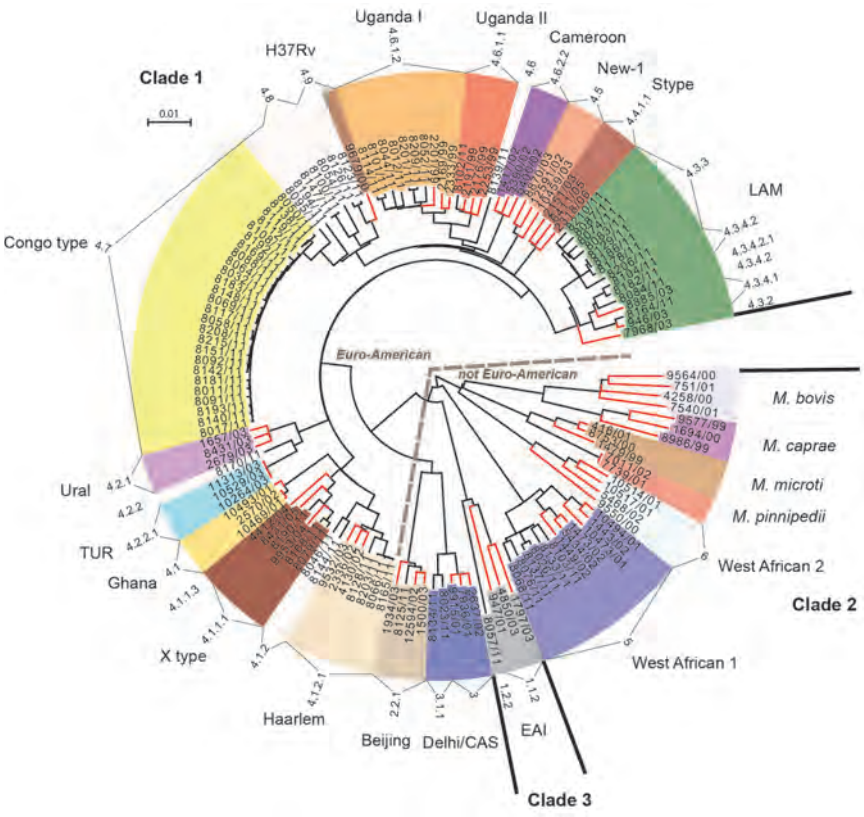

Figure 1. Maximum-likelihood tree of *Mycobacterium tuberculosis* complex isolates from Congo (black branch tips) and 65 reference strains (red branch tips). The tree was calculated by using the general time reversible substitution model with gamma distribution based on single-nucleotide polymorphisms identified by whole-genome sequencing. Models were tested and the tree generated by using MetaPiga software version 3.1 (*18*) and the maximum-likelihood ratio test. Midpoint rooting was performed. Distinct colors were chosen for the lineages identified; leaves with white background represent strains that initially were not assigned to particular lineages because of ambiguous typing patterns from mycobacterial interspersed repetitive unit, restriction fragment length polymorphism, or spoligo analysis (data not shown). The numerical code assigned to the respective lineages at the outer rim of the circular tree shows the Coll-nomenclature inferred from the whole-genome sequencing data. EAI, East African Indian; LAM, Latin American Mediterranean; TUR, Turkish. Scale bar indicates nucleotide substitutions per site.

likelihood tree (Figure 1) that shows that the Congo-type strains cluster most closely with H37Rv, presumably being part of a larger sublineage of H37Rv-related strains mentioned in other settings (*23*,*24*). Seven additional strains belonging to the Coll lineages 4.8 and 4.9 form a specific branch together with Congo type and H37Rv. We generated a tree containing bootstrap values supporting the branches of the tree (online Technical Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/23/3/16-0679-Techapp1.pdf).

Bayesian coalescent analysis approximated the last common ancestor of all Congo-type strains in our study to 1958 (95% highest posterior density 1947–1967). Thus, the Congo-type strains of this study probably emerged in the past 53 years.

WGS enabled us to identify SNPs specific for the Congo type by extracting the SNPs from the node specific for the Congo-type sublineage only and the common node for the Congo type and the more distant strain 8095/11. We found 49 SNPs unambiguously associated with the Congo-type sublineage (online Technical Appendix Table). The analysis of the pairwise distances revealed a homogenous population of the Congo-type strains with a median pairwise distance of 20 SNPs only (range 2–53 SNPs), whereas this distance was larger among the strains of other lineages (online Technical Appendix Figure 2).

The MDR isolates belonged to the Uganda and the Beijing lineages. By contrast, the strain resistant against INH and STR could be assigned to the Congo-type sublineage. We found no significant association between the Congo type and basic variables, such as sex, age, date of specimen collection, or patients' residence or HIV status.

Cluster Analysis

Among the strains in the study population, we identified 4 groups of strains with pairwise distances within 0–5 SNPs. Such close relationships between the strains indicate recent transmission. Two of the genome clusters are formed by Congo-type strains (2 clusters formed by 2 strains, 15% of the Congo-type strains). The other 2 genome clusters consisted of 2 isolates each, of either the LAM or Coll sublineage 4.8 closely related to H37Rv.

When we used a wider cluster definition of 12 SNPs maximum distance, 30 strains were grouped in 5 clusters ranging in size from 2 to 20 strains (Figure 2). Overall, 23 of the 26 Congo-type strains (88.5%) are in 1 large (n = 20) and 1 smaller (n = 3) cluster. Three (75%) isolates of the Haarlem lineage and 2 (17%) isolates of the LAM lineage are grouped in such clusters.

Discussion

Similar to other countries in Central Africa, Congo has a high incidence of TB. Our aim was to determine the population structure and transmission dynamics of the MTBC strains in Brazzaville.

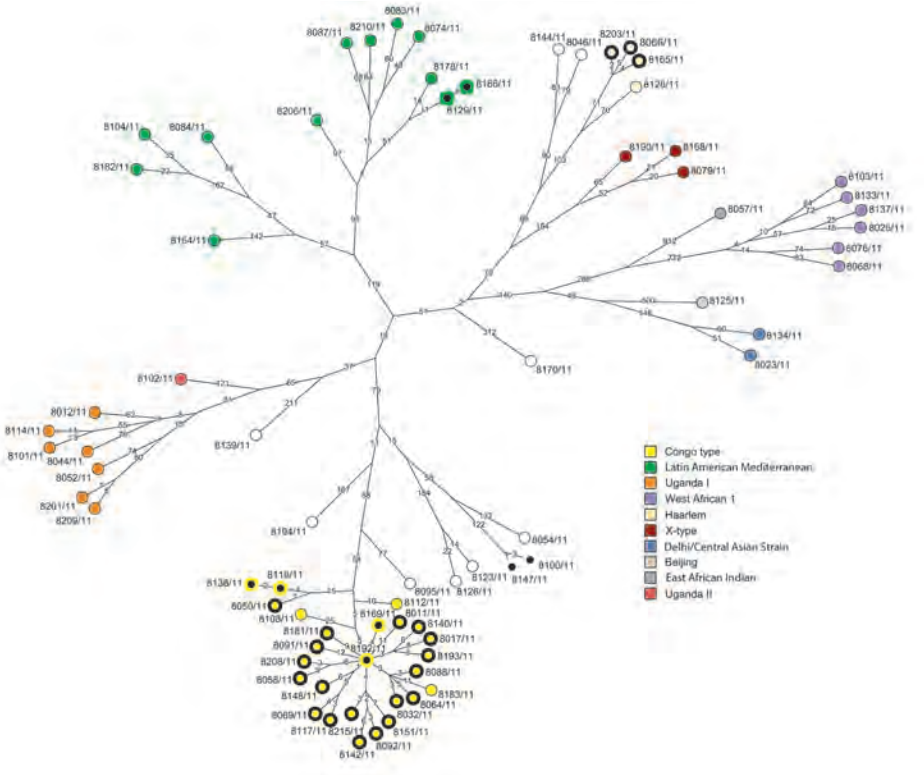

Figure 2. Maximum parsimony tree of *Mycobacterium tuberculosis* complex isolates from Congo. The tree was calculated on the basis of the concatenated single-nucleotide polymorphism (SNP) list. Branch labels indicate SNP distances; node labels represent the strain identifiers. Clusters based on pairwise comparisons were plotted on the tree: clusters ≤5 SNPs, nodes filled in black; clusters ≤12 SNPs, nodes with bold black outlines.

Our genotyping approach showed that all strains investigated were either *M. tuberculosis* or *M. africanum*; *M. tuberculosis* was most prevalent. These findings are in accordance with most recent studies from the other African countries reporting a predominance of *M. tuberculosis* strains (*25*–*28*). The high prevalence of *M. tuberculosis* detected in our investigation suggests that this predominance of *M. tuberculosis* strains might equally be the case for Congo, mainly driven by the newly described Congo-type sublineage.

The closely related strains of the Euro-American Congo-type sublineage were responsible for 35% of TB cases in the study population and showed a low pairwise genetic distance resulting in a high genome-based cluster rate, indicating ongoing recent transmission. It is tempting to speculate that strains from the Congo type are highly successful in the area and are recently expanding in the region of Brazzaville. On the other hand, strains of other MTBC lineages showed a higher degree of genetic diversity and formed smaller clusters with <3 strains. Such a high diversity is somewhat unexpected because, in a TB-endemic area, only few dominant clones, such as the Congo type, are hypothesized to circulate (*29*). The aforementioned findings point to a particular capacity of strains of the Congo type to spread in the area; for example, because of adaptation to the host population, as already postulated for other MTBC lineages (*5*,*30*). Accordingly, the diversity in the other MTBC lineages might reflect a higher rate of cases from reactivation of past TB infections, as suggested by a study conducted in South Africa with a high incidence of TB and high strain diversity (*31*).

The dominance of particular highly spreading clones, however, appears to be a more general phenomenon seen in several high-incidence areas. For example, strains of the Beijing lineage dominate in East Asia (*5*,*32*); the F11 *M. tuberculosis* genotype in Western Cape, South Africa (*31*); the LAM10-Cam family in Cameroon (*25*); and the K family, a sublineage of the Beijing genotype, in South Korea (*33*). In focusing on Africa, recent investigations revealed that Cameroon MTBC strains are responsible for most TB cases in several West Africa countries, such as Ghana and Cameroon (*25*,*27*); strains of the Uganda sublineage predominate in East Africa (*34*); and strains of the Sierra Leone sublineage predominate in Sierra Leone (*35*). Together, these data indicate marked differences in circulating mycobacterial strains in different Central Africa countries, suggesting a region-specific selection and spread of dominant sublineages of the Euro-American lineage.

Genome analysis enables not only high-resolution description of MTBC population diversity but also improved resolution of strains in recent transmission chains (*14*,*16*). Consistently, a SNP distance up to 5 SNPs was found in strains from confirmed direct human-to-human transmission, whereas a 12-SNP distance was proposed as a threshold to define larger cluster/transmission networks (*14*,*16*). Using these thresholds, we found that strains of the Congo-type sublineage formed a genetically homogenous

group with median pairwise genome SNP distances of 20 SNPs (range 2–53 SNPs) and a large number of Congo-type strains in clusters differing by ≤5 SNPs (15%) or a maximum of ≤12 SNPs (88.5%). This finding supports the presence of a larger transmission network of the Congo-type strains that presumably emerged in the past 53 years.

Only 2 of the 74 isolates characterized in this study were MDR, whereas recently Aubry et al. reported a higher MDR rate among MTBC isolates from Brazzaville and Pointe Noire (15 of 46 strains investigated; 10 strains belonging to the same lineage based on MIRU-VNTR and spoligotyping) (*36*). However, 7 of the 15 MDR strains in that study were obtained from retreated TB patients within a short 9-day sampling period. Even though we detected only 2 MDR strains of different lineages in the population in our study, the presence of MDR strains might pose a serious future challenge to public health authorities because these strains might have the potential to spread in the population; in line with this concern, 8 of the MDR strains described by Aubry et al. have been isolated from persons with newly diagnosed cases, which the authors suggested might reflect the transmission of an MDR clone (*36*). Thus, the development of drug-resistant phenotypes among particular lineages circulating in Congo, especially the successful Congo type, should be revisited as part of a larger investigation to evaluate the actual extent of MDR TB and potential pockets of transmission in Congo.

The data from our genome-based investigation of circulating MTBC strains in Congo demonstrate the presence of a new, predominant, and highly transmissible sublineage, the Congo type, which belongs to the Euro-American lineage. Larger molecular epidemiologic studies with respect to sociogeographic data and in addition to traditional contact tracing investigations will be required in Central Africa to gain a better understanding of recent transmission networks, the emergence of dominant lineages, and the prevalence of drug-resistant phenotypes in this wider geographic setting. Such studies may be implemented in large networks, such as the Central Africa Network on Tuberculosis, HIV/AIDS and Malaria (sponsored by the European and Developing Countries Clinical Trials Partnership), with the objective of conducting baseline investigations of TB in its members state: Gabon, Cameroon (*37*,*38*), and Congo (*6*).

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Whole-Genome Analysis of *Bartonella ancashensis*, a Novel Pathogen Causing Verruga Peruana, Rural Ancash Region, Peru

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The genus *Bartonella* contains >40 species, and an increasing number of these *Bartonella* species are being implicated in human disease. One such pathogen is *Bartonella ancashensis*, which was isolated in blood samples from 2 patients living in Caraz, Peru, during a clinical trial of treatment for bartonellosis. Three *B. ancashensis* strains were analyzed by using whole-genome restriction mapping and high-throughput pyrosequencing. Genome-wide comparative analysis of *Bartonella* species showed that *B. ancashensis* has features seen in modern and ancient lineages of *Bartonella* species and is more related to *B. bacilliformis*. The divergence between *B. ancashensis* and *B. bacilliformis* is much greater than what is seen between known *Bartonella* genetic lineages. In addition, *B. ancashensis* contains type IV secretion system proteins, which are not present in *B. bacilliformis*. Whole-genome analysis indicates that *B. ancashensis* might represent a distinct *Bartonella* lineage phylogenetically related to *B. bacilliformis*.

Bartonelloses are major emerging infectious bacterial diseases because of the high prevalence of chronic *Bartonella* infections in mammals and humans and their increasing risk for infection of immunocompromised populations (*1*–*4*). *Bartonella* species are present worldwide and are associated with several diseases, such as Carrion's disease, caused by *B. bacilliformis*; cat-scratch disease, caused by *B. henselae*; and trench fever, caused by *B. quintana* (*2*,*5*–*7*). *B. henselae* and *B. quintana* are also associated with chronic infections, such as bacillary angimatosis and endocarditis, and *B. quintana* chronic bacteremia is found in homeless populations worldwide (*3*,*8*–*12*).

In addition to these 3 major infectious pathogens, an increasing number of new *Bartonella* spp. have been identified in recent years as zoonotic pathogens transmitted by diverse arthropod vectors (*1*,*2*,*7*). Currently, >40 official and candidate *Bartonella* species are listed in the Taxonomy Database of the National Center for Biotechnology Information (http://www.bacterio.net/Bartonella.html); 31 are recognized species. During their evolution, *Bartonella* lineages have adapted to a variety of hosts and developed virulence factors associated with a diverse set of disease signs and symptoms (*13*–*15*).

Despite the high mortality rate for bartonellosis in Peru, studies on *Bartonella* pathogens are insufficient and mainly focused on *B. bacilliformis* (*16*). We previously reported identification of non-*bacilliformis Bartonella* in Peru during a clinical treatment trial (*17*–*19*). Using single-locus sequence typing, we identified 3 isolates (designated 20.00, 20.60, and 41.60) from 4 whole blood specimens collected from 2 patients (nos. 20 and 41) at day 0 or 60 during the clinical treatment trial in the Ancash region of Peru and found that these isolates had citrate synthase (*gltA*) gene sequences that diverged from that of *B. bacilliformis* reference strain KC583 (*17*–*19*). Multilocus sequencing typing and microbiological analyses indicated these 3 isolates are members of a novel *Bartonella* species, subsequently name *B. ancashensis* (*17*).

In this study, we performed genomic analyses of 3 non-*bacilliformis Bartonella* isolates obtained from patient blood samples (isolates 20.00, 20.60, and 41.60). Whole-genome analyses confirmed our previous identification of the isolates as a new species (*B. ancashensis*) (*20*) and identified unique genomic characteristics of *B. ancashensis*

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and differences between *B. ancashensis* and its closest relative (*B. bacilliformis*).

Methods

Ethics Statement

The human subject use protocol, including clinical specimen collection, and the consent procedure were approved in 2002 by the Institutional Review Boards of the Uniformed Services University of the Health Sciences (Bethesda, MD, USA); the Naval Medical Research Center Institutional Review Board (Bethesda, MD, USA); and the Universidad Peruana Cayetano Heredia (Lima, Peru). The trial details are available in the International Standard Randomized Controlled Trial Number registry (https://www.isrctn.com; trial no. ISRCTN16597283). Patients enrolled were 1–60 years of age, and written consent was obtained from the patient or the parent or guardian of the patient enrolled in the study.

Study Protocol

A clinical trial to compare rifampin, the standard drug for treatment of bartonellosis caused by chronic *B. bacilliformis* infection, with azithromycin, a possible alternative drug, was conducted in 2003 in the Caraz District of the Ancash region of Peru (Blazes DL, trial no. ISRCTN16597283). Patients with suspected chronic *B. bacilliformis* infection (verruga peruana) either came to the local hospital in Caraz or were identified by home visits. Patients (>1 year of age) with verruga peruana were randomly chosen to receive either a daily dose of rifampin (Pfizer, New York, NY, USA) for 14 days or 2 weekly doses of azithromycin (Pfizer) on days 0 and 7. For the patients who participated in the trial, survey data and medical records were collected on day 0 (baseline; time of presentation at the local hospital), and patients were then given the 2-week antimicrobial drug treatment. Clinical data was also collected on days 7, 14, 30, and 60.

In addition, peripheral blood specimens were collected from each patient on days 0, 7, 14, and 60 into tubes containing sodium citrate solution at the local hospital in Caraz and transported on ice to the clinical laboratory at the US Naval Medical Research Unit No. 6 (Lima, Peru) for blood cultures and analysis by PCR. Selected specimens, *Bartonella* isolates, and genomic DNA extracts were sent to the Naval Medical Research Center (Silver Spring, MD, USA) and the Walter Reed Army Institute of Research (Silver Spring, MD, USA) for additional investigations.

Blood specimens were cultured for ≥8 weeks as described (*17*–*19*). *Bartonella* culture-positive specimens were confirmed to be *Bartontella* species by using microbiological observations and molecular assays. Nucleic acids were isolated from culture-positive blood samples and subjected to PCR amplification of a 338-bp fragment of the *gltA* gene. The PCR product was sequenced by using the Sanger method.

We aligned partial *gltA* gene sequences (homologous to nt 781–1137 of the *B. bacilliformis* KC583 *gltA* gene) and used them for phylogenetic analyses. Samples with *gltA* sequences that showed major differences (>85% divergence) from those of *B. bacilliformis* were cultured on brain heart infusion agar supplemented with 10% defibrinated sheep blood (BD Diagnostics, Glencoe, MD, USA) for 10–28 days at the Naval Medical Research Center.

For next-generation sequencing (NGS), genomic DNA was extracted from *Bartonella* isolates, randomly fragmented by using focused ultrasonication (S2 System; Covaris, Inc., Woburn, MA, USA), and used in rapid shotgun genomic DNA library preparation and pyrosequencing with the 454 GS FLX Titanium System (Roche 454 Life Sciences, Branford, CT, USA). For whole-genome restriction map (WGRM) analysis, *Bartonella* isolates were freshly grown on brain heart infusion agar with 10% sheep blood at 30°C in an atmosphere of 5% CO_2 for 10–14 days. High molecular weight DNA was isolated by using the

Table 1. Characteristics of 4 non-*bacilliformis Bartonella* isolates from 2 patients with verruga peruana, rural Ancash region, Peru*

	Patient 20, 3-y-old boy, isolate no.		Patient 41, 10-y-old boy, isolate no.	
Characteristic	20.00	20.60	41.00	41.60
Patient signs	Lesions on hands and feet that disappeared after antimicrobial drug treatment		Lesions on hands and feet that disappeared after antimicrobial drug treatment	
Antimicrobial drugs used	Azithromycin on days 0 and 7		Rifampin daily on days 0–14	
Whole blood collection time	Day 0	Day 60	Day 0	Day 60
Peripheral blood smear†	Negative	Negative	Negative	Negative
Blood culture for *Bartonella* sp.	Positive	Positive	Positive	Positive
16S 321/533 TaqMan qPCR‡	Negative	Negative	3.93×10^5 (19.24)	6.36×10^4 (22.15)
16S 27F2/533R PCR	Negative	Negative	Positive	Positive
B. ancashensis–specific PCR§	Negative	Negative	Positive	Positive
Blood culture *gltA* PCR/sequencing	*B. ancashensis*	*B. ancashensis*	B. *bacilliformis*	*B. ancashensis*
Isolate by pure-culture sequencing	*rrs*, *gltA*, *rpoB*; whole genome	*rrs*, *gltA*, *rpoB*; whole genome	*gltA*	*rrs*, *gltA*, *rpoB*; whole genome

*_gltA_, citrate synthase gene; _rpoB_, β subunit of RNA polymerase gene; _rrs_, 16S rRNA gene.
†Rapid microscopic diagnosis for Carrion's disease (*16*).
‡Values are 16S rRNA gene copy number/microliter (quantitative cycle threshold).
§Primers 22RC-3F (5′-TTCGGCTTAGCTTATCCGTTTCACAA-3′) and 32RC-5R (5′-CGTAAGAGCTTTGTGGCAAAATAGCAA-3′) were used; the expected PCR amplicons size was 0.8 kb, which corresponds to nt 673839–674636 of *B. ancashensis* (GenBank accession no. CP010401).

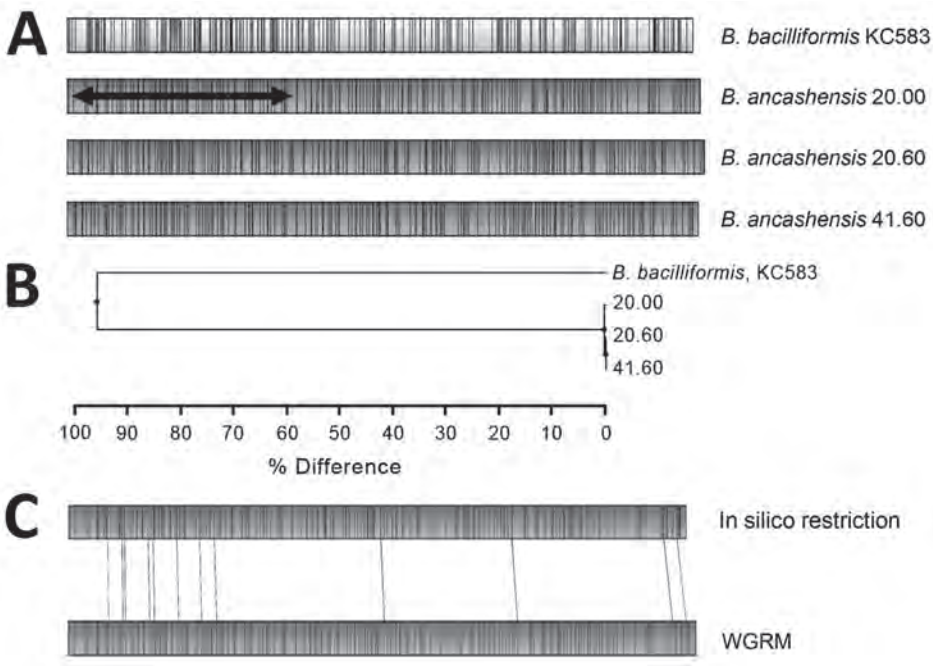

Figure 1. Alignment of whole-genome restriction maps and in silico map for *Bartonella* isolates from patients with verruga peruana, rural Ancash region, Peru. A) Maps for *B. ancashensis* isolates 20.00, 20.60, and 41.60 were determined by using optical mapping. Shaded areas indicate regions of alignment, unshaded areas indicate regions where restriction maps do not align, and black horizontal arrow indicates restriction sites. B) Phylogeny based on map similarity constructed by using the unweighted pair group method with arithmetic mean. C) Alignment of map for *B. ancashensis* predicted in silico from the complete genome sequence with the experimentally observed *B. ancashensis* map. WGRM, whole-genome restriction map.

Argus Sample Preparation Kit (OpGen Inc., Gaithersburg, MD, USA). DNA quality and quantity were assessed by using the Argus QCard Kit and mapped by using the Argus MapCard Kit and Argus Enzyme Kit-AflII with the Argus System (OpGen Inc.). Downstream analysis, clustering, and genome alignment was performed by using MapSolver version 3.2.4. (OpGen Inc.).

We de novo assembled NGS data into sequence contigs by using GS Assembler software version 2.5.3 (Newbler; https://wikis.utexas.edu/display/bioiteam/GS+De+novo+assembler) and then assembled the contigs to scaffolds with the WGRM as the physical reference. PCR amplification and open reading frame annotation were used to complete genome assembling. After we virtually digested the complete genome sequence with *Afl*II, we aligned the in silico whole-genome restriction map with the WGRM to ensure the correct order and orientation of the final assemblies (*21*).

We performed pairwise genome-wide comparative analysis by comparing homologous proteins from pairs of *Bartonella* species. Results are presented as density distribution curves for amino acid identity and as dot plots for pairwise amino acid identity for each homologous protein. Genes of interest were subjected to additional analysis, including gene cluster comparisons. For genome-wide phylogenetic analysis of *Bartonella* species, complete genome sequences or assembly contigs of whole genome sequences were aligned by using Mauve version 2.3.1 (*22*) to identify single nucleotide changes in conserved genomic regions. A total of 12,740 single-nucleotide polymorphisms were found in in 25.2 kb of sequence common to all 38 *Bartonella* strains. A phylogenetic tree was constructed by using the R phangorn package (*23*). The initial tree was constructed by using the neighbor-joining algorithm and optimized by using the parsimony maximum-likelihood method. Tree stability was evaluated by using 100 bootstrap replicates.

Results

In the clinical trial testing the efficacy of rifampin and azithromycin for treatment of chronic bartonellosis, blood specimens from 72 of 127 patients were positive for *Bartonella* species by culture, and *gltA* gene sequencing indicated that these patients were infected with only *B. bacilliformis*; however, 2 patients (nos. 20 and 41) were infected with *B. ancashensis* (*17–19*). DNA extracts from the 4 original whole blood specimens from these patients (20.00, 20.60, 41.00, and 41.60) were tested by using quantitative bacterial 16S rDNA PCR, standard bacterial 16S PCR, and *B. ancashensis*–specific PCR (Table 1). Whole blood from patient 20 was PCR negative for bacteria on days 0 and 60. However, high levels of bacteremia were seen for whole blood specimens from days 0 and 60 for patient 41.

Although levels of bacteremia differed greatly, clinical signs and symptoms for both patients were indistinguishable from each another and from those for other patients with confirmed cases of chronic *B. bacilliformis* infection. In addition, our results confirm that *B. ancashensis* was isolated from whole blood specimens of patient 41 on day 60, but not on day 0. The evidence suggests emergence of a novel *Bartonella* species in Peru that can cause its own verruga peruana–like infection in humans or possibly co-infect humans in conjunction with *B. bacilliformis*. It is intriguing that the

Table 2. *Bartonella* spp. strains (n = 41) used for whole-genome analysis of human pathogens causing verruga peruana, rural Ancash region, Peru*

Species	Strain	GenBank accession no.
B. alsatica	IBS 382	AIME01000000
B. ancashensis	20.00	NZ_CP010401
B. australis	Aust/NH1	NC_020300.1
B. bacilliformis	INS	AMQK01000000
	KC583	NC_008783.1
B. birtlesii	IBS 325	AKIP01000000
	LL-WM9	AIMC01000000
B. bovis	91-4	AGWA01000000
	m02	AGWB01000000
B. clarridgeiae	73	NC_014932.1
B. doshiae	NCTC 12862	AILV01000000
	ATCC 700133	JAGY01000000
B. elizabethae	F9251	AIMF01000000
	Re6043vi	AILW01000000
B. grahamii	as4aup	CP001562.1
B. henselae	Houston-1	BX897699.1
	JK 53	AHPI01000000
	Zeus	AHPJ01000000
B. koehlerae	C29	AHPL01000000
B. melophagi	K-2C	AIMA01000000
B. queenslandensis	AUST/NH15	CALX01000000
B. quintana	JK 31	AHPG01000000
	JK 63	AHPF01000000
	JK 67	AHPC01000000
	JK 68	AHPD01000000
	RM-11	CP003784.1
B. rattaustraliani	AUST/NH4	CALW02000000
B. rattimassiliensis	15908	AILY01000000
B. rochalimae	ATCC BAA-1498	FN645455.1–FN645467.1
	BMGH	AHPK01000000
Bartonella sp. DB5-6	DB5-6	AILT01000000
Bartonella sp. OS02	OS02	CALV01000000
B. tamiae	Th239	AIMB01000000
	Th307	AIMG01000000
B. taylorii	8TBB	AIMD01000000
B. tribocorum	CIP 105476	AM260525.1
B. vinsonii	OK-94-513	AILZ01000000
	Pm136co	AIMH01000000
	Winnie	NC_020301.1
B. washoensis	085-0475	AILX01000000
	Sb944nv	AILU01000000

*NA, not available.

bacteremia profile, based on blood cultures, for patient 41 changed from *B. bacilliformis* at day 0 to *B. ancashensis* at day 60.

We performed genome-wide analysis of the 3 *B. ancashensis* isolates (20.00, 20.60, and 41.60) by using the WGRM and NGS (*21*,*24*). The WGRMs of the 3 isolates showed >99.7% similarity with each other and <10% similarity to the WGRM of *B. bacilliformis* KC583. WGRM showed that the *B. ancashensis* genome is circular and ≈1.46 Mb. A region of ≈0.64 Mb in the 20.00 genome was inverted when compared with maps for isolates 20.60 and 41.60 (Figure 1).

The complete genome sequence of *B. ancashensis* strain 20.00 (GenBank accession no. NZ_CP010401) is circular (1,466,048 bp) and has a G + C content of 38.4%. These values are similar to those for the complete genome sequence of *B. bacilliformis* KC583 (NC_008783.1) (1,445,021 bp and a G + C content of 38.2%). The inverted region of the 20.00 genome (0.64 Mb) is flanked by two 507-bp repetitive sequences in an opposite orientation. The repetitive sequence is 97% identical to the gene for *B. bacilliformis* integrase (protein family HMM PF00589), a DNA breaking–rejoining enzyme that catalyzes DNA recombination.

We used whole-genome phylogenetic analyses to examine the evolutionary relatedness between *B. ancashensis* and 40 other *Bartonella* strains (Table 2; Figure 2). This analysis, based on 12,740 single-nucleotide polymorphisms in genomic sequences conserved across all species, showed that *B. ancashensis* isolate 20.00 is most closely related to *B. bacilliformis*, *B. bovis*, and *B. melophagi*. The degree of similarity between *B. ancashensis* and 15 other *Bartonella* species was further examined by assessing the pairwise similarity of homologous protein with the basic local alignment search tool score ratio method (*25*) (Figure 3, panel

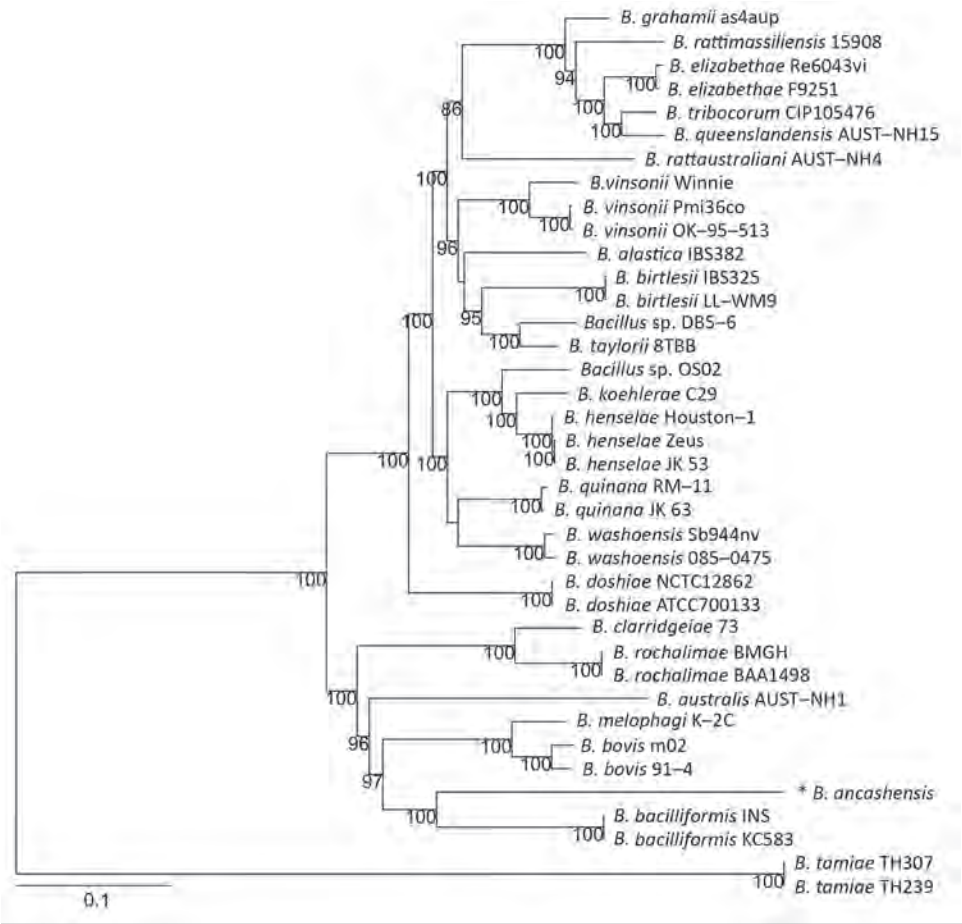

Figure 2. Phylogenetic relationship of *Bartonella ancashensis* isolates from patients with verruga peruana, rural Ancash region, Peru, with other *Bartonella* species based on whole-genome phylogeny. The tree is based on single-nucleotide polymorphisms identified in genomic regions common to all *Bartonella* strains examined. The initial tree was constructed by using the neighbor-joining algorithm and was optimized by using the parsimony maximum-likelihood method. Tree stability was evaluated by using 100 bootstrap replications. Asterisk indicates strain isolated in this study. Numbers along branches are bootstrap values. Scale bar indicates nucleotide substitutions per site.

A). *B. ancashensis* predicted proteins are most similar to *B. bacilliformis* proteins, but there are several distinctive differences. Specifically, 63.7% of *B. ancashensis* genes are part of a core genome common to all 15 comparator species, 10.7% of *B. ancashensis* proteins did not have a homolog in any of the reference *Bartonella* spp., and 89.3% of *B. ancashensis* proteins are present in ≤1 other *Bartonella* spp.; 79.1% of *B. ancashensis* proteins were found in *B. bacilliformis* (Figure 3, panel B).

Most *B. ancashensis* genes identified by pan-genome analyses had homologs in ≥1 *Bartonella* species (Figure 3, panel B). Eight protein-coding genes in *B. ancashensis* and *B. bacilliformis* were not present in any other species. An additional 5 protein-coding genes were present in *B. ancashensis*, *B. bacilliformis*, and *B. australis*, but in no other known species. In contrast, there were 8 protein-coding genes in *B. ancashensis* and all 14 species, excluding *B. bacilliformis*, but including *B. australis*, which is considered the most divergent *Bartonella* species currently recognized. Another 129 *B. ancashensis* protein-coding genes have homologs in only a subset of the non-*bacilliformis* *Bartonella* (*26*).

In 3 regions of the *B. ancashensis* genome, the similarity between *B. bacilliformis* and *B. ancashensis* proteins was lower than the average value (Figure 4). Proteins in variable region 1 (genes 30–90) are homologous to non-*bacilliformis* species proteins, including phage proteins, such as HigA and HigB. Proteins encoded in variable region 2 (genes 180–240) are absent from *B. bacilliformis*. Among these proteins are those that have high similarity with *Bartonella* type IV secretion system proteins, which are hypothetical gene products that have moderate identity with proteins from other non-*bacilliformis* species, and novel hypothetical proteins. Variable region 3 (genes 620–704) contains loci encoding hypothetical proteins not seen in other species and several toxin proteins that are not found in *B. bacilliformis*, including the RelE/StbE replicon stabilization toxin, the RelB/StbD replicon stabilization protein, and the HigB toxin protein.

Analysis identified 2 characteristic features of *B. ancashensis*: type IV secretion complex (VirB2) proteins, which are not found in *B. bacilliformis*; and flagella proteins, which are not found in *Bartonella* species in lineage 4, including human pathogens *B. quintana* and *B. henselae* (*15*,*27*,*28*). In isolates 20.60 and 41.60, the 31 flagellar genes encoded by *B. ancashensis* are located in the identical order and distances as their homologs in the *B. bacilliformis* genome. Isolate 20.00 has a large genomic

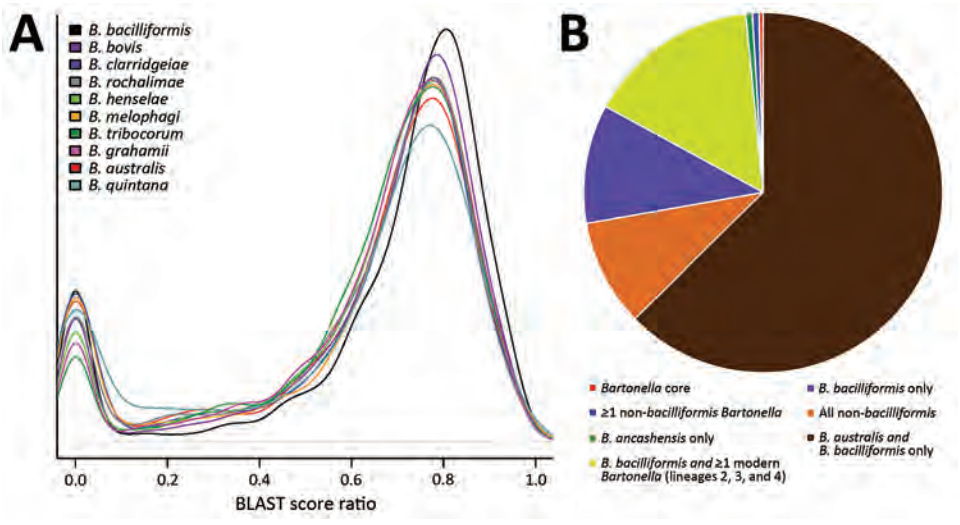

Figure 3. Proteomic analysis of *Bartonella ancashensis* isolated from patients with verruga peruana, rural Ancash region, Peru, and 10 related *Bartonella* species. A) Density plot showing similarity between *B. ancashensis* protein-coding genes and genes from 10 of the more closely related *Bartonella* species. Similarity scores are based on the BLAST score ratio method (BSR) (*25*). A score of 1.0 indicates identity between 2 proteins, and a score <0.3 indicates that the 2 proteins do not show meaningful similarity. The *Bartonella* species whose protein similarity score distribution has a peak closest to 1 (*B. bacilliformis*) has the highest overall protein similarity to *B. ancashensis*. Horizontal gray line indicates density = 0. B) Presence of homologous protein-coding genes in *B. ancashensis* and 15 *Bartonella* species. Proteins from 2 species with a BSR score ≥0.3 were considered to be homologs and present in *B. ancashensis* and ≥1 *Bartonella* species.

inversion, and this rearrangement results in 1 gene (*FliJ*) required for production of flagella arranged in a reversed orientation and separated from the other genes of the main flagellar gene cluster. In isolate 20.00, *FliJ* is ≈600 kb from the flagellar gene cluster; in isolates 20.60 and 41.60, *FliJ* is ≈100 kb from this cluster (Figure 5).

A *Leptospira* species virulence attenuation study identified a group of paralogous virulence modulated (VM) genes, which are believed to play a role in human pathogenesis caused by *Leptospira interrogans* (*29*). Subsequent comparative genomic analysis showed that VM proteins are present in other bacterial pathogens, including *B. bacilliformis* and *B. australis*; *B. ancashensis* encodes 5 VM proteins (Figure 6). In contrast, no homologs of VM proteins were found by a basic local alignment search tool search in any other recognized *Bartonella* species. As seen for the VM proteins of *Leptospira* species, VM protein genes in these 3 *Bartonella* species were scattered throughout their genomes, and the number of VM proteins was different for each species.

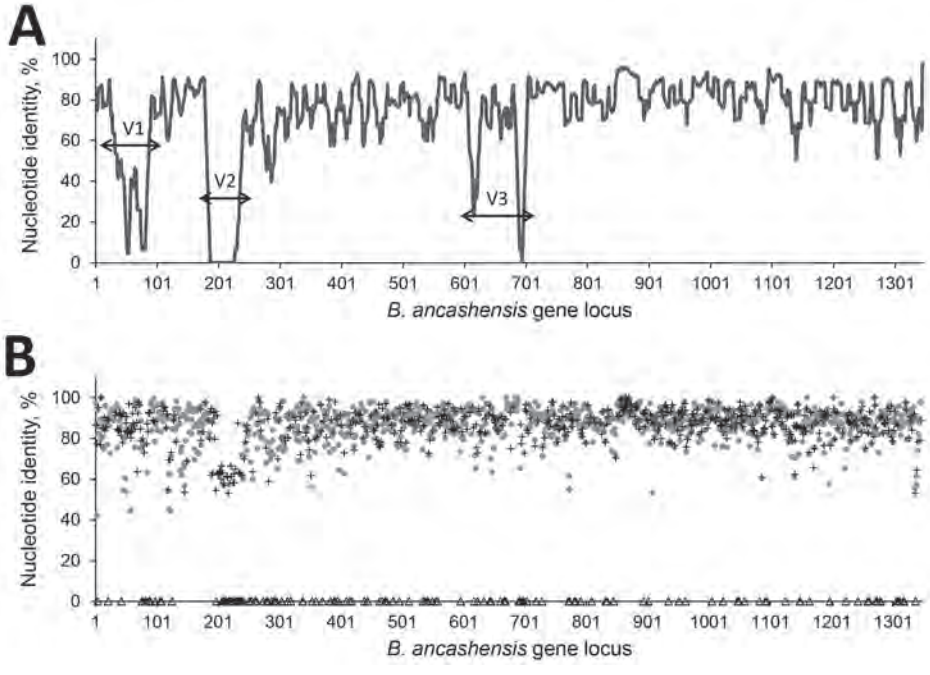

Figure 4. Pairwise comparisons of protein-coding genes of *Bartonella* isolates from patients with verruga peruana, rural Ancash region, Peru. A) Nucleotide similarity of *B. ancashensis* protein-coding sequences compared with those of *B. bacilliformis* (gray circles in panel B), top hit non-*bacilliformis Bartonella* (+ in panel B) and not determined hypothetic proteins (Δ in panel B). B) Nucleotide similarity plot. Average nucleotide identity within a window of 10 genes was plotted against gene locus number. Regions I, II, and III are 3 variable regions that contain genes with lower nucleotide identities or absent in the *B. bacilliformis* genome. V, variable.

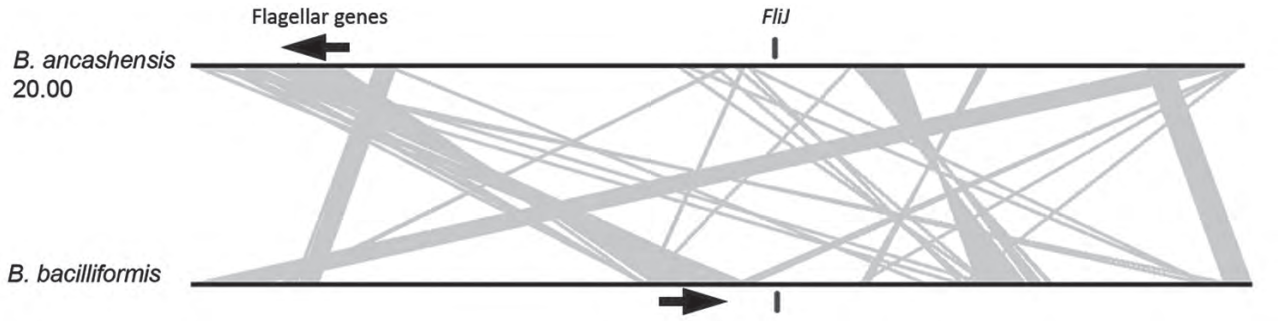

Figure 5. Genetic arrangement of the genome of *B. ancashensis* isolate 20.00 from a patient with verruga peruana, rural Ancash region, Peru, compared with that of *B. bacilliformis* KC583. Black lines indicate chromosomes and gray lines link syntenic genomic regions that are rearranged between the 2 genomes. *FliJ* genes are indicated by black vertical bars, and flagellar gene clusters are indicated by arrows, which indicate direction of transcription.

Discussion

B. bacilliformis was discovered in Peru in 1907 as the causative agent for Oroya fever and verruga peruana. Since then, *B. bacilliformis* has been the primary subject in bartonelloses studies in South America. However, it has long been speculated that multiple *Bartonella* spp. might be circulating in this region (*30*). Our study and previous work clearly indicate *B. ancashensis* is a unique *Bartonella* species that cocirculates with *B. bacilliformis* in the Ancash region of Peru, where the prevalence of *B. bacilliformis* is high (*16*,*31*,*32*).

Moreover, *B. ancashensis* has several unique genomic features. Like *B. bacilliformis*, this species encodes flagellar

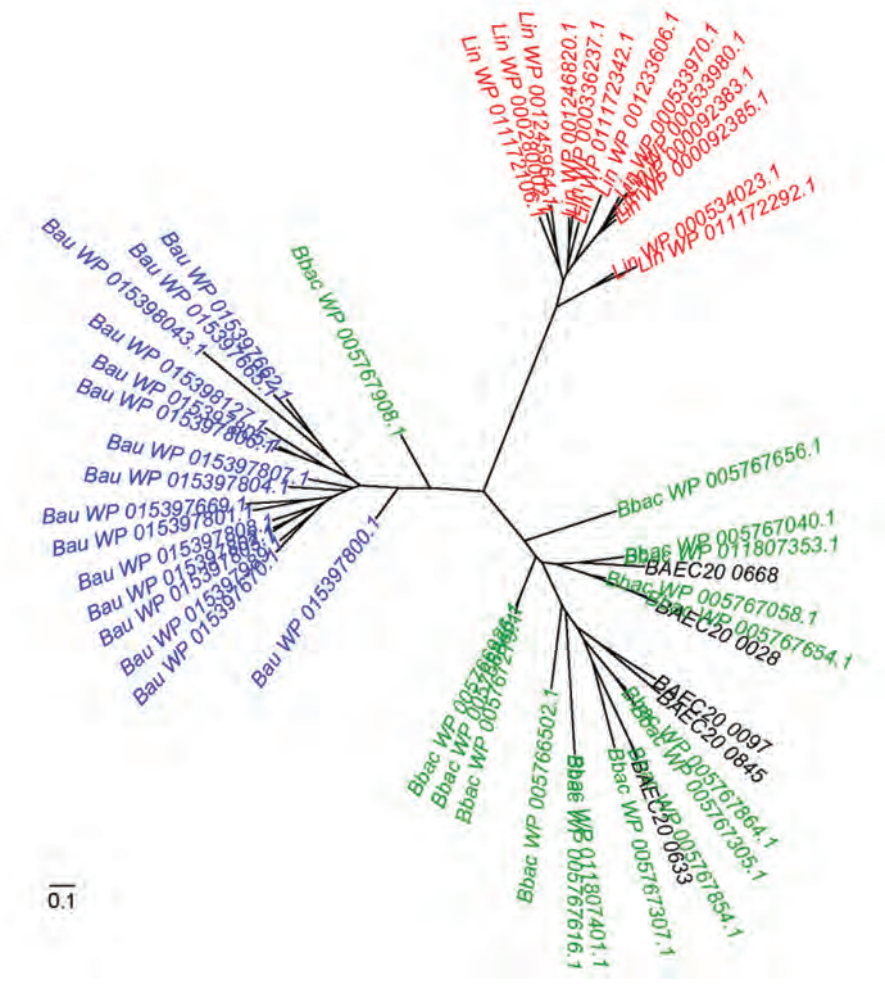

Figure 6. Virulence-modulating (VM) proteins in *Leptospira* and *Bartonella* species. Unrooted phylogenetic tree of VM proteins from *Leptospira interrogans* (*Lin*, red), *B. australis* (*Bau*, blue), *B. bacilliformis* (*Bbac*, green), and *B. ancashensis* (*BAE*, black). VM proteins from *L. interrogans*, *B. bacilliformis*, and *B. australis*, cluster by species; the 5 VM proteins encoded by *B. ancashensis* group with their *B. bacilliformis* homologs. Scale bar indicates amino acid substitutions per site.

genes, which are believed to be essential for erythrocyte invasion, the main route of pathogenesis caused by *B. bacilliformis* (*33*). In contrast, human pathogens *B. quintana* and *B. henselae* do not produce flagella, and host-specific pathogenicity is believed to be linked to the type IV secretion system (T4SS) gene clusters VirB and Trw (*27*,*28*,*33*,*34*). *B. ancashensis* and 2 other pathogens (*B. clarridgeiae* and *B. rochalimae*) have flagellar and VirB T4SS gene clusters (*15*,*26*,*27*).

Whole-genome analysis of 3 *B. ancashensis* isolates showed that isolate 20.00 differs from the other 2 isolates by a large genomic region inversion. Future comparative analysis of gene expression profiles in these strains will show whether this genomic inversion alters the regulation of flagellar genes, as well as other virulence factors within or adjacent to the inverted region.

Moreover, it will be useful to investigate whether the genomic inversion (isolate 20.00 vs. 20.60) is related to administration of antimicrobial drugs to infected patients because isolates 20.60 and 41.60 were obtained from patients after they received antimicrobial drugs and cleared any clinical signs or symptoms of bartonellosis because infection persisted 60 days after enrollment in the study and administration of antimicrobial drugs. Furthermore, these isolates were previously tested for their in vitro susceptibility to rifampin and azithromycin and both were found to be susceptible (*17*). However, in vitro antimicrobial drug susceptibility testing for *Bartonella* spp. has been largely limited in its clinical utility. However, *B. ancashensis* might be capable of producing chronic asymptomatic infections that could be caused by its unique genomic characteristics.

The VM genes belong to a family of homologous virulence-related genes originally identified in *L. interrogans* and modulate the pathogenesis of *L. interrogans* in humans (*29*). These genes in *B. bacilliformis*, *B. australis*, and *B. ancashensis*, but not in other *Bartonella* spp., the further comparative analysis and functional studies on these VM proteins and the large number of other hypothetical proteins in *Bartonella* spp. will shed light on the pathogenesis mechanisms of bartonellosis, which are so far largely unknown.

Intensified tropical disease surveillance and advances in scientific methods led to an increasing number of new *Bartonella* species being identified in recent years (*30*,*35*). These studies identified 1 major phylogenetic lineage of the genus *Bartonella*. Our study and other genomic studies demonstrated that *B. bacilliformis*, which was historically regarded as the ancestral *Bartonella* spp., probably diverged from other species in the distant past and evolved as a species uniquely adapted to the human host because no small mammals have been implicated as reservoir hosts for *B. bacilliformis* (*15*,*26*,*27*,*36*). Although *B. ancashensis* is a novel species most closely related to *B. bacilliformis*, it has a nucleotide divergence of ≈20% when compared with *B. bacilliformis* for conserved genomic regions, which is exceedingly high and comparable with distances among proposed *Bartonella* lineages (*15*,*26*). Therefore, it is rational to designate *B. ancashensis* as an independent lineage parallel to the *B. bacilliformis* lineage. Our study provided evidence that there might be more *Bartonella* species and subspecies in regions of South America.

Bartonellosis has affected humans for hundreds to thousands of years, remains endemic to several areas, and continues to cause sporadic outbreaks in many regions. Identification of a novel *Bartonella* species in this study not only provided long-awaited evidence of species diversity in areas to which *B. bacilliformis* is endemic but also indicates the need for acquisition of sufficient genomic data, which will enable pathogenomics studies. Such studies will make essential contribution to a comprehensive understanding and effective control of bartonelloses.

Dr. Mullins is laboratory medicine fellow at the University of Maryland, Baltimore, MD. Her primary research interest is development of infectious disease diagnostic assays.

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Epidemiology of Nontuberculous Mycobacterial Lung Disease and Tuberculosis, Hawaii, USA

Jennifer Adjemian, Timothy B. Frankland, Yihe G. Daida, Jennifer R. Honda, Kenneth N. Olivier, Adrian Zelazny, Stacey Honda, D. Rebecca Prevots

Previous studies found Hawaiians and Asian-Americans/Pacific Islanders to be independently at increased risk for nontuberculous mycobacterial pulmonary disease (NTMPD) and tuberculosis (TB). To better understand NTM infection and TB risk patterns in Hawaii, USA, we evaluated data on a cohort of patients in Hawaii for 2005–2013. Period prevalence of NTMPD was highest among Japanese, Chinese, and Vietnamese patients (>300/100,000 persons) and lowest among Native Hawaiians and Other Pacific Islanders (50/100,000). Japanese patients were twice as likely as all other racial/ethnic groups to have *Mycobacterium abscessus* isolated (adjusted odds ratio 2.0, 95% CI 1.2–3.2) but were not at increased risk for infection with other mycobacteria species. In contrast, incidence of TB was stable and was lowest among Japanese patients (no cases) and highest among Filipino, Korean, and Vietnamese patients (>50/100,000). Substantial differences exist in the epidemiology of NTMPD by race/ethnicity, suggesting behavioral and biologic factors that affect disease susceptibility.

The incidence of nontuberculous mycobacterial pulmonary disease (NTMPD) is increasing in North America and many parts of the world (*1*–*7*), whereas the incidence of tuberculosis (TB), caused by *Mycobacterium tuberculosis*, has decreased in industrialized countries during the same period (*8*,*9*). However, population-based data are lacking from areas with high incidence of NTMPD and TB.

In 2012, the first-ever US nationwide analysis on the prevalence of NTMPD among older adults found that Hawaii had the highest prevalence of any state, 4 times the national average (*1*). Another recent study identified Hawaii as having the highest age-adjusted mortality rate from NTMPD (*10*). In these reports, persons identified as Asian American/Pacific Islander were at increased risk for NTMPD, independent of geographic area of residence (*1*). Similarly, Hawaii has a TB incidence greater than the national average (*11*), and among Native Hawaiians and Other Pacific Islanders (NHOPI), this rate is 13 times higher than among non-Hispanic whites. Moreover, 67% of all TB patients and 96% of Asians with TB are foreign-born (*12*); because Hawaii has a large population of foreign-born Asians, it is disproportionately affected (*13*).

The high risk for NTMPD (*1*,*14*) and TB (*11*,*12*) in Hawaii enables us to estimate their relative prevalence in the same population and identify distinct and overlapping risk factors in subpopulations. Because Hawaii has the highest proportion of Asian and NHOPI residents of any state (41% and 9%, respectively) (*15*), this population provides an opportunity to better describe the epidemiology of these diseases in a high-risk setting.

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Materials and Methods

Study Population

We extracted electronic medical record data on Kaiser Permanente Hawaii (KPH) patients enrolled for ≥9 months during 2005–2013. We queried databases for all patients with acid-fast bacilli (AFB) smears or mycobacterial cultures performed on respiratory specimens. For each specimen tested, we extracted collection date, body site of collection, AFB smear results, and mycobacterial culture results, including species identified if positive. For patients tested for mycobacteria, we extracted data on selected co-morbidities using codes from the International Classification of Diseases, Ninth Revision (ICD-9). We extracted data on age, sex, self-identified race/ethnicity, and residential zip code for all patients. Racial/ethnic data are based on self-report at enrollment; patients could select ≥1 of 28 options, including 23 Asian and Pacific Islander subgroups. This study was approved by the National Institutes of Health Office for Human Subjects Research and the KPH Institutional Review Board.

Laboratory Analysis

We used standard laboratory procedures in a Clinical Laboratory Improvement Amendments–certified laboratory for mycobacterial testing by AFB smear and culture. We used commercially available probes at KPH for identification of *M. tuberculosis* complex and *M. avium* complex (MAC); we sent other isolates to Associated Regional and University Pathologists Laboratories for further species identification by PCR or sequence-based identification. Laboratory methods were consistent across the entire study period. We recorded all mycobacteria species identified; *M. gordonae* was considered nonpathogenic and excluded from case definitions (*16*).

Case Definitions

We defined 3 case groups. The nontuberculous mycobacteria (NTM) isolation group comprised patients with ≥1 pathogenic pulmonary NTM species identified; the NTMPD group comprised patients with ≥2 positive cultures for pathogenic NTM identified; and the TB group comprised patients with ≥1 pulmonary *M. tuberculosis* isolate, regardless of their NTM isolation status.

Data Analysis

We calculated annual and overall period prevalence for each case group by using the KPH source population as the denominator. We stratified all estimates by age group, sex, race/ethnicity, and island (for those residing in a single zip code throughout the study). We used Poisson regression models with allowance for overdispersion and an offset given by the log of the KPH population to calculate the annual percentage change in prevalence over time. We used χ^2 and Student *t* tests to test for differences in case frequencies by demographic factors and co-morbidities. We assessed associations with NTM infection and TB in logistic regression models by using a Firth penalized likelihood option to reduce small-sample bias, where all smear-positive patients were compared with patients not identified as positive. For models evaluating demographic factors, the negative comparison group included those testing negative and those not tested, assuming they were negative. For models evaluating co-morbidities, the negative comparison group included only those testing negative for mycobacteria. To better assess the independent effect of race/ethnicity, we limited regression models to patients with a single racial/ethnic group identified. We adjusted all models for sex, age group, and years present in the KPH database (range 1–9 years). We further adjusted models identifying notable demographic variables by co-morbidities that might be associated with smoking (i.e., chronic obstructive pulmonary disease [COPD], as determined by ICD-9 codes documented for emphysema, obstructive chronic bronchitis, or

Table 1. Demographic characteristics of study population for investigation of mycobacterial infection prevalence, by mycobacterial testing status and culture result, Hawaii, 2005–2013*

Characteristic	KPH population	NTM isolated			TB isolated	Culture negative
		Total	1 positive culture	≥2 positive cultures		
Total	373,168 (100)	455 (0.1)	201 (0.05)	254 (0.07)	40 (0.01)	1,707 (0.5)
Female sex	184,292 (49)	245 (54)	100 (50)	145 (57)	12 (30)	797 (47)
Age, mean ± SD, y	36 ± 22	66 ± 16	65.2 ± 6.3	66 ± 16	55 ± 16	62 ± 17
Island of residence†						
Oahu	196,391 (69)	300 (79)	135 (81)	165 (78)	21 (58)	1,061 (78)
Maui	61,427 (21)	56 (15)	22 (13)	34 (16)	13 (36)	220 (16)
The Big Island	26,052 (9)	22 (6)	10 (6)	12 (6)	2 (6)	74 (5)
Kauai	2,452 (1)	0	0	0	0	0
Years in KPH						
1	72,661 (19)	12 (3)	6 (3)	6 (2)	3 (8)	89 (5)
2–4	118,403 (32)	59 (13)	32 (16)	27 (11)	10 (25)	321 (19)
≥5	182,104 (49)	384 (84)	163 (81)	221 (87)	27 (68)	1,297 (76)
Race/ethnicity‡						
White	124,966 (43)	172 (38)	76 (38)	96 (38)	4 (10)	685 (40)
Black	6,260 (2)	2 (0.4)	2 (1)	0	0	31 (2)
NHOPI	90,785 (24)	81 (18)	46 (23)	35 (14)	5 (13)	415 (24)
Asian	142,931 (38)	265 (58)	110 (55)	155 (61)	30 (75)	908 (53)
Filipino	60,314 (21)	90 (20)	34 (17)	56 (22)	22 (55)	306 (18)
Japanese	38,571 (13)	92 (20)	40 (20)	52 (20)	0	325 (19)
Chinese	23,932 (8)	50 (11)	20 (10)	30 (12)	2 (5)	190 (9)
Korean	5,967 (2)	19 (4)	9 (4)	10 (4)	2 (5)	39 (2)
Vietnamese	2,030 (0.7)	5 (1)	2 (1)	3 (1)	1 (3)	13 (0.8)
Other	22,801 (8)	20 (4)	9 (4)	11 (4)	3 (8)	83 (5)
>1 Race/ethnicity	105,159 (36)	111 (24)	58 (29)	53 (21)	6 (15)	476 (28)

*Values are no. (%) patients unless otherwise indicated. A total of 2,197 patients had pulmonary mycobacterial culture results (see Table 2). KPH, Kaiser Permanente Hawaii; NHOPI, Native Hawaiian and Other Pacific Islander; NTM, nontuberculous mycobacteria; NTMPD, nontuberculous mycobacterial pulmonary disease; TB, tuberculosis.
†Only includes KPH patients residing in a single ZIP code throughout the study period (n = 286,322).
‡Some patients self-reported >1 racial/ethnic category; percentage calculated out of total patients reporting race/ethnicity (n = 292,336).

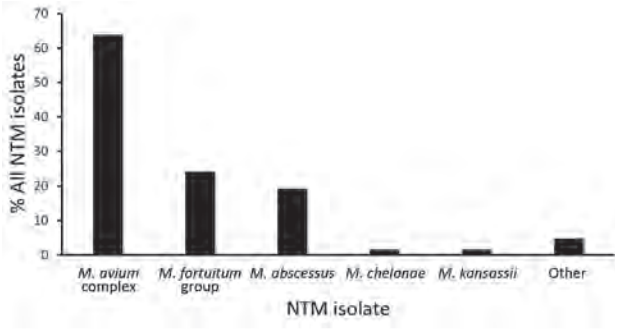

Figure 1. Occurrence of nontuberculous mycobacteria species identified from pulmonary specimens obtained among a cohort of Kaiser Permanente Hawaii patients, Hawaii, 2005–2013. Other pathogenic nontuberculous mycobacteria species identified (n = 21) were *Mycobacterium flavescens, M. immunogenum, M. mucogenicum, M. neoaurum, M. scrofulaceum*, *M. simiae*, and undifferentiated *M. chelonae/abscessus*. NTM, nontuberculous mycobacteria.

chronic airway obstruction), for those with co-morbidity data available. We conducted analyses by using SAS version 9.3 (SAS Institute, Inc., Cary, NC, USA) and calculated adjusted odds ratios (aORs) and 95% CIs.

Results

During 2005–2013, a total of 373,168 patients were enrolled in KPH, representing nearly one third of the Hawaii population (*15*); the demographic distribution of our study population was similar to that for the state, with slightly more white patients (Table 1) (*15,17*). Of the patient total, 2,197 (0.6%) had ≥1 mycobacterial culture performed on a respiratory specimen; 1,086 (49%) of those had only 1 culture performed (range 1–29 cultures/patient). Of patients who had culture performed, 455 (21%) had pathogenic NTM isolated: 201 (44%) had 1 positive culture, and 254 (56%) had ≥2 positive cultures (NTMPD cases) (Table 1). The most frequently isolated species were MAC (n = 290; 64%), *M. fortuitum* group (n = 109; 24%), and *M. abscessus* (n = 87; 19%) (Figure 1); 91 (20%) patients had >1 NTM species identified. A higher proportion of patients with *M. abscessus* isolated (30%) were positive for ≥2 years in the database, compared with those with MAC (16%) or *M. fortuitum* group (6%).

Compared with the overall KPH population (Table 1), NTM infection patients were significantly older (mean age ± SD 66 ± 16 years vs. 36 ± 22 years; p<0.05), and a greater proportion were female (54% vs. 49%), enrolled in KPH for ≥5 years (84% vs. 49%), and self-identified as Asian (58% vs. 38%), whereas significantly fewer (p<0.05) were NHOPI (18% vs. 24%) or self-identified as being >1 race/ethnicity (24% vs. 36%). NTMPD patients were similar in age, sex, and racial/ethnic distribution to those with only 1 NTM-positive culture (Table 1).

Of the 455 patients who had a mycobacterial culture performed, 40 (2%) had positive results for *M. tuberculosis*. TB patients were younger (mean age ± SD 55 ± 16 years) and a greater proportion were male (n = 28; 70%) compared with the KPH population. Among TB patients, 30 (75%) self-identified as Asian, 5 (13%) as NHOPI, and 4 (10%) as white; 6 (15%) self-identified as >1 race/ethnicity. Of the 40 TB patients, 5 (13%) were co-infected with NTM (all with MAC and 1 additionally with *M. fortuitum* group).

Prevalence of NTM Isolation

The annual prevalence of NTM isolation more than doubled over time, from 20 cases/100,000 persons in 2005 to 44 cases/100,000 persons in 2013 (annual percentage change 6%, 95% CI 1%–11%; p = 0.01). NTMPD prevalence also doubled, from 9 to 19 cases/100,000 persons, although this increase was not significant (p = 0.2) (Figure 2, panel A). When evaluated by species, this trend was observed for MAC only (Figure 2, panel B).

The 2005–2013 period prevalence for NTM isolation was 122 cases/100,000 persons. Prevalence was 4-fold greater among persons ≥65 years of age than for those 50–64 years of age (696 vs. 183 cases/100,000 persons) (Figure 3). For persons ≥75 years of age, period prevalence was 906 cases/100,000 persons, and within this age group, prevalence was substantially higher among those enrolled in KPH for ≥5 years (1,049 cases/100,000 persons) compared with those enrolled for only 2–4 years (577 cases/100,000 persons). NTM isolation period prevalence was highest on Oahu (153 cases/100,000 persons), followed by Maui (91 cases/100,000 persons) and Hawaii (the Big Island) (84 cases/100,000 persons); no cases were identified on other islands.

NTM isolation period prevalence was highest among Japanese, Chinese, Korean, and Vietnamese patients (≈300 cases/100,000 persons; average annual prevalence 34 cases/100,000 persons), similar among Filipino and white patients (162 and 156 cases/100,000 persons, respectively; average annual prevalence 18 cases/100,000 persons), and lowest among NHOPI patients (50 cases/100,000 persons; average annual prevalence 6 cases/100,000 persons) (Figures 4, 5). NTM isolation prevalence was progressively greater by increasing age group across nearly all racial/ethnic groups evaluated (Figure 5). Among Vietnamese and Korean patients, the highest NTM isolation rates were observed among those 50–64 years of age (767 and 823 cases/100,000 persons, respectively); however, these estimates did not significantly differ from those observed among persons ≥65 years of age in these populations (p>0.2). Sex differences in NTM isolation prevalence were also noted by racial/ethnic group (Figure 6). Among Vietnamese patients, NTM isolation was more prevalent among men than women (568 vs. 105 cases/100,000 persons), whereas

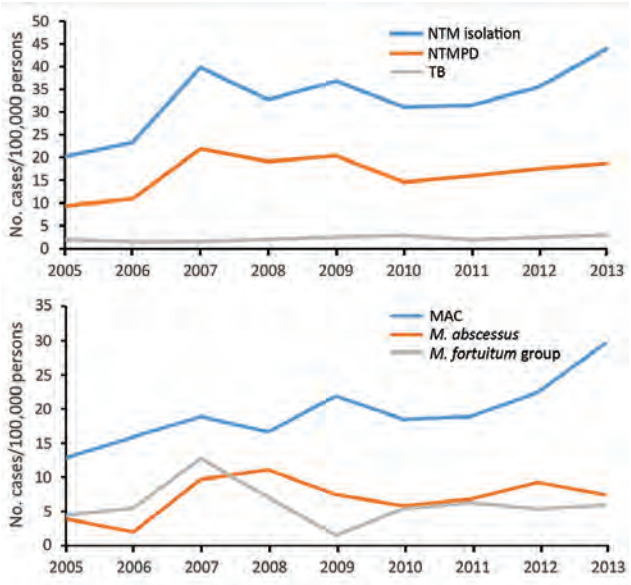

Figure 2. Annual prevalence of pulmonary nontuberculous mycobacteria isolation, nontuberculous mycobacterial pulmonary disease, and tuberculosis (A) and annual prevalence of pulmonary nontuberculous mycobacteria isolation by species (B) among a cohort of Kaiser Permanente Hawaii patients, Hawaii, 2005–2013. MAC, *Mycobacterium avium* complex; NTM, nontuberculous mycobacteria; NTMPD, nontuberculous mycobacterial pulmonary disease; TB, tuberculosis.

among Japanese patients, NTM isolation was more prevalent among women than men (378 vs. 287 cases/100,000 persons). For all other racial/ethnic groups, prevalence did not differ greatly by sex. NTM isolation prevalence was consistently double the NTMPD prevalence.

TB Incidence

TB incidence was stable over time (≈2 cases/100,000 persons/year) (Figure 2, panel A). The cumulative 9-year rate was 11 cases/100,000 persons and was higher among men than women (15 vs. 7 cases/100,000 persons). The overall TB rate was highest among those ≥50 years of age and did not differ between those 50–65 years of age (24 cases/100,000 persons) and those ≥65 years of age (30 cases/100,000 persons) (Figure 3). TB incidence was highest on Maui (21 cases/100,000 persons), followed by Oahu (11 cases/100,000 persons) and the Big Island (8 cases/100,000 persons).

Among racial/ethnic groups, the TB rate was lowest among Japanese (no cases), white, and NHOPI patients (3 cases/100,000 persons for both) and highest among other patients of Asian ethnicities (33 cases/100,000 persons) (Figure 4). Most (55%) TB patients were Filipino, with an overall incidence of 52 cases/100,000 persons; although only 2 (5%) TB patients were Korean and 1 (3%) Vietnamese, their overall incidence was similarly high (53 and 60 cases/100,000 persons, respectively) (Figure 4).

Co-morbid Conditions

Of the co-morbidities evaluated (Table 2), the most frequently reported conditions among NTM infection patients were COPD (41%) and bronchiectasis (37%). Bronchiectasis was more frequent among patients with *M. abscessus* infection (58%) than those with MAC (39%) or *M. fortuitum* group (31%) infection, whereas COPD was more frequent among patients with MAC infection (45%) than those with *M. abscessus* (38%) or *M. fortuitum* group (36%) infection. Patients with NTMPD had a similar co-morbidity profile. For TB patients, few co-morbidities were reported, although COPD (13%) was most common. Although only 4 KPH patients with cystic fibrosis were tested for mycobacteria in this cohort, 2 (50%) had NTMPD.

Species-Specific Risk Analysis

We obtained logistic regression results by mycobacterial species adjusted for sex, age, and years in KPH (Table 3). Compared with all other KPH patients, NHOPI patients were at decreased risk for NTM infection (aOR 0.5, 95% CI 0.3–0.9), whereas an increased risk for NTM infection and TB was observed among Asian patients, particularly those who were Vietnamese (NTM, aOR 3.7, 95% CI 1.6–8.6; TB, aOR 9.6, 95% CI 2.0–46.8), Korean (NTM, aOR 1.9, 95% CI 1.1–3.4; TB, aOR 5.9, 95% CI 1.7–20.4), and Filipino (NTM, aOR 1.3, 95% CI 1.3, 1.0–1.7; TB, aOR 8.8, 95% CI 4.9–16.0). However, differences in risk were noted by NTM species (Table 3). Japanese patients were nearly twice as likely to have *M. abscessus* infection (aOR 2.0, 95% CI 1.2–3.2) but were not at increased risk for MAC or *M. fortuitum* group infection compared with other racial/ethnic groups. Filipino patients also were at increased risk for *M. abscessus* (aOR 2.0, 95% CI 1.2–3.3) and MAC (aOR 1.5, 95% CI 1.1–2.1) infection. Vietnamese patients were more likely to have MAC (aOR 3.7, 95% CI 1.3–10.6), *M. fortuitum* group (aOR 8.7, 95% CI 3.0–25.0), and *M. abscessus* (aOR 5.0, 95% CI 1.0–24.6)

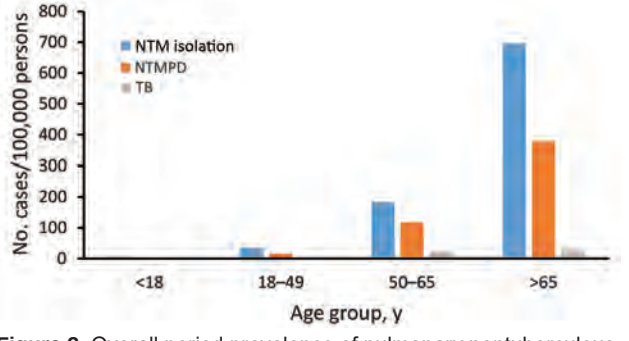

Figure 3. Overall period prevalence of pulmonary nontuberculous mycobacteria isolation, nontuberculous mycobacterial pulmonary disease, and tuberculosis, by age group, among a cohort of Kaiser Permanente Hawaii patients, Hawaii, 2005–2013. NTM, nontuberculous mycobacteria; NTMPD, nontuberculous mycobacterial pulmonary disease; TB, tuberculosis.

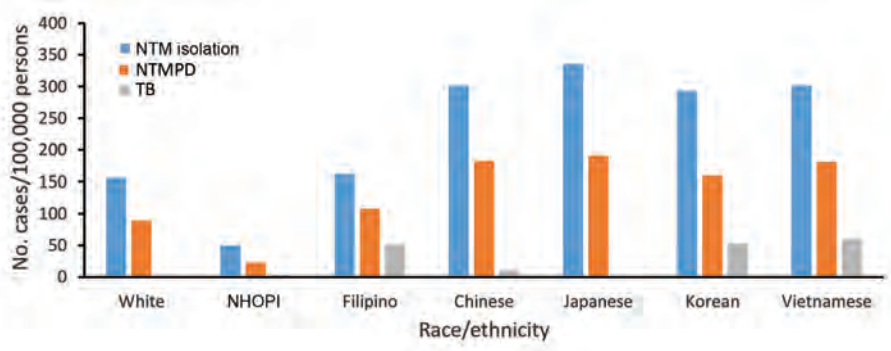

Figure 4. Overall period prevalence of pulmonary nontuberculous mycobacteria isolation, nontuberculous mycobacterial pulmonary disease, and tuberculosis, by race/ethnicity, among a cohort of Kaiser Permanente Hawaii patients, Hawaii, 2005–2013. NHOPI, Native Hawaiians and Other Pacific Islanders; NTM, nontuberculous mycobacteria; NTMPD, nontuberculous mycobacterial pulmonary disease; TB, tuberculosis.

infection. Korean patients were only more likely to have *M. fortuitum* group infection (aOR 4.0, 95% CI 1.7–9.5). A substantially higher risk for co-infections with multiple NTM species was observed for only Vietnamese patients (aOR 17.3, 95% CI 5.9–50.6).

NTM infection patients were more likely to have bronchiectasis, whereas only patients with MAC infection were additionally more likely to have COPD (p<0.001) (Table 3). After also adjusting for COPD, Asian patients remained at increased risk for NTM infection (aOR 1.4, 95% CI 1.2–1.7), whereas white patients were at decreased risk (aOR 0.8, 95% CI 0.6–1.0). Similarly, Japanese (aOR 1.9, 95% CI 1.1–3.0) and Filipino (aOR 1.7, 95% CI 1.0–2.9) patients remained at increased risk for *M. abscessus* infection, as did Vietnamese (aOR 6.0, 95% CI 1.8–19.6) and Korean (aOR 3.9, 95% CI 1.6–9.7) patients for *M. fortuitum* group infection. After controlling for COPD status, Japanese patients were no longer more likely to have *M. fortuitum* group infection, and only Filipino patients (aOR 1.4, 95% CI 1.0–2.0) remained at increased risk for MAC infection.

Discussion

We identified significant epidemiologic trends and species-specific differences in the prevalence of NTM infection in Hawaii. By using patient laboratory data from a representative population of Hawaii residents enrolled in a closed healthcare system, we found that the prevalence of NTM infection was double that previously reported (*1*). However, epidemiologic differences by species and race/ethnicity were noted within Hawaii.

Among persons living in Hawaii, Asians are at greater risk for both NTM infection and TB compared with other racial/ethnic groups, although this varied by racial/ethnic subgroup and mycobacterial species. Japanese patients were at increased risk for *M. abscessus* infection only, whereas Vietnamese and Korean patients were at a substantially higher risk for both *M. fortuitum* group infection and TB compared with others in Hawaii. Filipino patients were at increased risk for *M. abscessus* infection, MAC infection, and TB. In contrast, NHOPI patients were less likely to have NTM infection than all others. Nonetheless, all non-Asian populations evaluated, including whites and NHOPIs, had a higher estimated NTMPD prevalence than has been reported elsewhere in the country (*1*,*2*,*18*–*20*).

The increased prevalence of NTM infection in Hawaii might be attributable to unique environmental conditions. Soils are high in humic acid, a component associated with higher numbers of mycobacteria (*14*,*21*–*23*), which might contribute to an increased potential for environmental exposure through not just soil but also water sources, because soil is often the source of waterborne pathogens (*24*), and both can result in the generation of bioaerosols that might contain mycobacteria (*25*). Our previous studies identified factors related to a greater persistence of moisture droplets in the air, including higher saturated vapor pressure and evapotranspiration rates, to be associated with a greater risk for NTM infection (*14*,*26*). Additional, systematic environmental sampling is needed to speculate further on exposure sources.

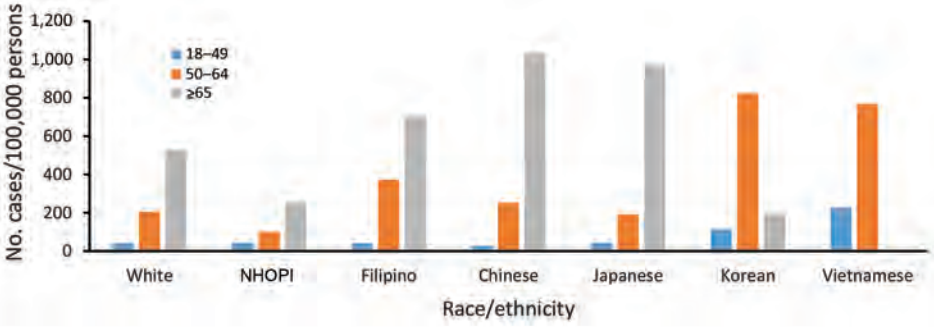

Figure 5. Overall period prevalence of pulmonary nontuberculous mycobacteria isolation, by race/ethnicity and age group, among a cohort of Kaiser Permanente Hawaii patients, Hawaii, 2005–2013. No cases of nontuberculous mycobacteria isolation were reported among Vietnamese patients ≥65 years of age. NHOPI, Native Hawaiians and Other Pacific Islanders.

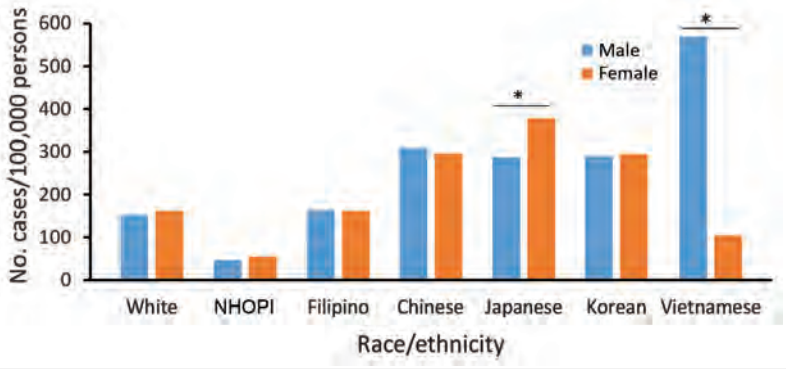

Figure 6. Overall period prevalence of pulmonary nontuberculous mycobacteria isolation, by race/ethnicity and sex, among a cohort of Kaiser Permanente Hawaii patients, Hawaii, 2005–2013. Prevalence reflects number of unique patients with pulmonary nontuberculous mycobacteria detected during the 9-year period. *p<0.05 (significant difference). NHOPI, Native Hawaiians and Other Pacific Islanders.

Higher rates of NTM infection have been reported in East Asian populations, although global studies are limited (*27*). In 2005, the annual prevalence of NTM infection in Japan was estimated to be 33–65 cases/100,000 persons (*28*), which is similar to the rate identified in our present study. NTM infection was also frequently reported among hospital patients in South Korea (*29*). However, a study from Taiwan estimated an annual prevalence of NTM infection of 8 cases/100,000 persons in 2008 (*30*), which is markedly less than what we observed in Hawaii. Assessing the role of race/ethnicity in mycobacterial lung disease is complex because these categorizations likely reflect behavioral, cultural, and biologic factors (*31*).

An increasing trend was detected only for MAC infection; infection rates with all other NTM species remained relatively stable over time. A similar increasing trend in NTM infection prevalence was reported among US Medicare patients, although in that study species-level data were unavailable (*1*). It is impossible to know if this rise in prevalence is attributable to greater environmental exposure or host susceptibility or is in part attributable to greater clinical awareness and detection; however, the latter seems unlikely given that an increase was only observed for 1 species.

In contrast to NTM, annual rates of TB were substantially lower than what has been reported for Hawaii (*12*). This finding is likely attributable to differences in risk among the KPH population compared with all Hawaiians with TB. The difference was especially notable for NHOPIs (*33*), who in this population had TB incidence rates similar to that of whites. However, even among KPH patients, the incidence of TB was 5 times higher among Vietnamese, Korean, and Filipino patients compared with the overall population.

Different co-morbidity patterns were observed by mycobacteria species. Although all NTM infection patients were more likely to have bronchiectasis regardless of species, only patients with MAC infection were additionally more likely to have COPD. After controlling for COPD status along with other factors in models, the association between MAC infection and Vietnamese race/ethnicity was no longer significant, potentially reflecting behavioral contributions to their increased risk (assuming COPD is a proxy for smoking status) (*33*). In fact, Vietnamese and Korean NTM infection patients were similar to TB patients in terms of their age and sex, with a higher proportion of middle-aged persons affected and, for Vietnamese patients, more male than female patients. However, estimates from persons ≥65 years of age were limited for Vietnamese and Korean patients because of smaller population sizes, making lower observed rates compared with those aged 50–65 possibly an artifact of fewer opportunities to detect NTM.

Table 2. Occurrence of selected co-morbid conditions among Kaiser Permanente Hawaii patients who had mycobacterial cultures performed, Hawaii, 2005–2013*

	No. (%)							
		NTM isolated						
Co-morbidity	Total, n = 2,197†	NTM, n = 455	MAC, n = 290	*M. abscessus*, n = 87	*M. fortuitum*, n = 109	NTMPD, n = 254	TB, n = 40	Culture negative, n = 1,707
COPD	780 (36)	188 (41)	130 (45)	33 (38)	39 (36)	108 (43)	5 (13)	588 (34)
Bronchiectasis	335 (15)	169 (37)	114 (39)	50 (58)	34 (31)	113 (44)	1 (3)	165 (10)
Cystic fibrosis	4 (0.2)	2 (0.4)	1 (0.3)	1 (1)	0 (0)	2 (0.8)	0 (0)	2 (0.1)
HIV	24 (1)	4 (0.9)	3 (1)	0 (0)	1 (0.9)	4 (2)	0 (0)	20 (1)
Coccidiomycosis	12 (0.6)	4 (0.9)	2 (0.7)	0 (0)	1 (0.9)	1 (0.4)	0 (0)	8 (0.5)
Sarcoidosis	10 (0.5)	3 (0.7)	2 (0.7)	0 (0)	1 (0.9)	0 (0)	0 (0)	7 (0.4)
Malignant neoplasm of trachea, bronchus, or lung	472 (21)	56 (12)	34 (12)	8 (9)	12 (11)	27 (11)	0 (0)	416 (24)
Other malignancies	453 (21)	92 (20)	62 (21)	16 (18)	20 (18)	52 (20)	5 (13)	357 (21)

*COPD, chronic obstructive pulmonary disease; MAC, *Mycobacterium avium* complex; NTM, nontuberculous mycobacteria; NTMPD, NTM pulmonary disease; TB, tuberculosis.
†Total number and percentage of patients with co-morbidity out of all Kaiser Permanente Hawaii patients with pulmonary mycobacterial culture results.

Table 3. Risk for nontuberculous mycobacterial pulmonary disease (by mycobacteria species) or tuberculosis among Kaiser Permanente Hawaii patients, by demographic characteristics and co-morbid condition, Hawaii, 2005–2013*

Characteristic	aOR (95% CI)				
	NTM	*M. abscessus*	MAC	*M. fortuitum* group	TB
Racial/ethnicity					
White	0.9 (0.7–1.1)	0.7 (0.4–1.1)	1.0 (0.7–1.2)	1.0 (0.6–1.5)	**0.2 (0.05–0.6)**
NHOPI	**0.5 (0.3–0.9)**	0.1 (0.01–1.4)	**0.4 (0.2–0.9)**	0.5 (0.2–1.3)	0.5 (0.1–2.6)
Black	0.6 (0.1–3.1)	1.1 (0.7–16.4)	1.0 (0.2–4.8)	0.7 (0.05–11.5)	NA
Asian	**1.4 (1.2–1.7)**	**2.5 (1.7–3.9)**	**1.4 (1.1–1.8)**	1.5 (1.0–2.2)	**4.9 (2.6–9.2)**
Filipino	**1.3 (1.0–1.7)**	**2.0 (1.2–3.3)**	**1.5 (1.1–2.1)**	1.3 (0.7–2.1)	**8.8 (4.9–16.0)**
Japanese	1.2 (1.0–1.6)	**2.0 (1.2–3.2)**	1.0 (0.7–1.4)	**1.6 (1.0–2.5)**	NA
Chinese	1.3 (0.9–2.0)	1.9 (0.9–3.9)	1.5 (0.95–2.3)	0.3 (0.06–1.5)	1.0 (0.2–5.0)
Korean	**1.9 (1.1–3.4)**	2.0 (0.6–7.0)	1.4 (0.6–3.2)	**4.0 (1.7–9.5)**	**5.9 (1.7–20.4)**
Vietnamese	**3.7 (1.6–8.6)**	**5.0 (1.0–24.6)**	**3.7 (1.3–10.6)**	**8.7 (3.0–25.0)**	**9.6 (2.0–46.8)**
Island of residence					
Oahu	**1.5 (1.2–1.9)**	**2.6 (1.4–5.2)**	**1.6 (1.2–2.2)**	1.3 (0.8–2.0)	0.6 (0.3–1.2)
Maui	**0.7 (0.6–1.0)**	**0.4 (0.2–0.9)**	0.7 (0.5–1.0)	0.8 (0.5–1.4)	**2.3 (1.2–4.3)**
Hawaii	**0.6 (0.4–1.0)**	0.5 (0.2–1.4)	0.6 (0.4–1.1)	0.9 (0.5–2.0)	0.7 (0.2–2.3)
Age group, y					
<18	Referent	Referent	Referent	Referent	Referent
18–49	**7.4 (2.9–19.3)**	1.9 (0.3–11.3)	**7.8 (2.2–27.9)**	**8.3 (1.6–42.7)**	3.9 (0.7–21.0)
50–64	**31.2 (12.2–79.7)**	**22.1 (4.4–111.8)**	**32.7 (9.3–114.6)**	**26.8 (5.3–135.5)**	**13.4 (2.6–69.1)**
≥65	**106.4 (42.0–270.0)**	**65.1 (13.0–325.1)**	**127.1 (36.6–441.3)**	**61.4 (12.2–308.1)**	**16.6 (3.1–88.5)**
Sex					
M	Referent	Referent	Referent	Referent	Referent
F	1.0 (0.9–1.3)	1.4 (0.9–2.1)	1.1 (0.9–1.4)	0.8 (0.6–1.2)	**0.4 (0.2–0.8)**
Years in KPH					
1	Referent	Referent	Referent	Referent	Referent
2–4	**2.6 (1.4–4.7)**	1.1 (0.4–2.9)	**2.7 (1.1–6.3)**	**7.0 (1.3–36.3)**	1.7 (0.5–5.7)
≥5	**6.4 (3.6–11.2)**	2.3 (0.96–5.4)	**7.7 (3.5–16.8)**	**14.9 (3.0–74.2)**	2.3 (0.8–6.9)
Co-morbid condition†					
Bronchiectasis	**8.3 (6.5–10.7)**	**12.0 (7.6–18.8)**	**7.0 (5.2–9.2)**	**4.6 (2.9–7.1)**	0.4 (0.09–2.2)
COPD	**1.8 (1.4–2.2)**	1.3 (0.8–2.0)	**1.9 (1.5–2.5)**	1.5 (1.0–2.3)	0.4 (0.2–1.1)
Coccidiomycosis	**4.5 (1.4–15.1)**	2.0 (0.1–38.4)	3.5 (0.8–15.2)	4.0 (0.7–23.4)	2.8 (0.2–50.1)
Sarcoidosis	2.0 (0.6–7.2)	1.2 (0.1–22.8)	2.2 (0.5–9.4)	3.5 (0.6–20.0)	3.0 (0.2–54.6)
Malignant neoplasm‡	**0.7 (0.5–0.9)**	**0.5 (0.2–1.0)**	0.6 (0.4–0.9)	0.7 (0.4–1.3)	**0.07 (0.01–1.0)**
Other malignancies	**0.7 (0.5–0.8)**	**0.5 (0.3–0.8)**	**0.7 (0.5–0.9)**	0.7 (0.5–1.1)	**0.3 (0.1–0.8)**

*aOR, adjusted odds ratio; COPD, chronic obstructive pulmonary disease; KPH, Kaiser Permanente Hawaii; MAC, *Mycobacterium avium* complex; NHOPI, Native Hawaiian and Other Pacific Islander; NTM, nontuberculous mycobacteria; Ref, referent group; TB, tuberculosis; NA, not available because limited sample size resulted in unstable model estimates. All aORs and 95% CIs associated with each racial/ethnic group, island, and co-morbid condition were assessed independently as a binary variable via logistic regression models, adjusted for age, sex, and years in KPH. Estimates for years in KPH, age, and sex were obtained from a single adjusted multivariable model. Race/ethnicity was self-reported; race/ethnicity-specific analyses were limited to patients reporting only 1 racial/ethnic group. Statistical significance (p<0.05) is indicated with boldface font.
†Data only available for KPH patients with a mycobacterial culture performed; percentages listed are out of total patient population with that co-morbidity.
‡Of trachea, bronchus, or lung.

Japanese NTM infection patients, on the other hand, were more likely to be female, older, and have *M. abscessus* infection compared with other racial/ethnic groups. Previous studies have reported racial/ethnic disparities in smoking rates in Hawaii, with Japanese and Chinese persons reporting significantly lower rates of nicotine dependence than other Asian groups, which might explain some of the trends and associations we observed (*34*).

Differences in prevalence were also noted by island, with NTM infection patients more likely to reside on Oahu. NTM environmental exposure levels may vary by island, perhaps because of differences in island ecology or variations in water sources and distribution systems. Differences in exposure might also be related to factors associated with residing in a more urban area such as Honolulu. However, even within the KPH system, patients living in closer proximity to more specialized healthcare facilities are probably more likely to be tested for mycobacteria, given symptoms. Similarly, even after controlling for age, longer enrollment time in KPH was associated with a substantially higher risk for NTM infection. The association with enrollment time could be a proxy for increased duration of exposure to NTM in Hawaii, but it could also reflect greater access to healthcare over a longer period of time. Regardless of whether this increased risk for NTM infection is attributable to greater environmental exposure or just increased access to care, the dose-response effect observed between KPH enrollment time and NTMPD risk demonstrates that the prevalence estimates we generated probably reflect underestimates of the actual burden of NTM infection in Hawaii, given that half the patients were enrolled for <4 years.

This study is subject to several limitations. These findings reflect the epidemiology of mycobacterial infections in patients participating in the KPH system and might not be representative of those without access to similar

healthcare plans, who likely differ socioeconomically. Although income data on KPH patients were unavailable, previous studies have shown higher prevalence of NTM infection in higher-income areas, likely because of greater access to diagnostic and clinical services (*14*). Similarly, because most KPH patients reside in Oahu, we might be unable to generalize these findings to other islands, especially those with limited KPH representation. Additional studies are needed to better identify interisland differences in mycobacterial infection risk. We were also unable to assess actual time of exposure in Hawaii because data on length of time residing in Hawaii were unavailable. Additionally, co-morbidity data were based on ICD-9 codes; therefore, actual co-morbidity status might have been misclassified for some patients. Last, some patients classified as negative in our models might have been in fact positive for NTM infection but were not tested and identified in our dataset; however, because the NTM infection is rare, this would probably affect very few patients and have limited effect on our results. Despite these limitations, differences in prevalence by race/ethnicity were striking, underscoring the importance of genetic, environmental, and behavioral contributions to risk for NTM infection.

In conclusion, we identified differences in the epidemiology of pulmonary mycobacterial infection and disease in Hawaii residents by racial/ethnic group. The prevalence of NTM infection and TB are far greater in Hawaii than elsewhere in the United States, probably because of a combination of increased environmental exposure and a possibly more susceptible population, attributable in part to its unique demographic profile. In particular, Asians living in Hawaii are at significantly higher risk for NTM infection and TB compared with other racial/ethnic groups, whereas NHOPIs appear to be at decreased risk compared with all others. Additional prospective studies assessing genetic, behavioral, and environmental risk factors in high-risk regions such as Hawaii are needed to better understand the role of race/ethnicity in mycobacterial lung disease.

Author contributions were as follows: study concept and design, J.A., R.P., J.H.; acquisition of data, J.A., T.F., Y.D., J.H., K.O., A.Z., S.H., R.P.; analysis and interpretation of data, J.A., R.P., A.Z.; drafting of the manuscript, J.A.R.P., J.H., Y.D., T.F., S.H., A.Z., K.O.; critical revision of the manuscript for important intellectual content, J.A., R.P., J.H., Y.D., T.F., S.H., A.Z., K.O.; statistical analysis, J.A.; study supervision, J.A., R.P., S.H.

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Mycobacterium tuberculosis Transmission among Elderly Persons, Yamagata Prefecture, Japan, 2009–2015

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In many countries with low to moderate tuberculosis (TB) incidence, cases have shifted to elderly persons. It is unclear, however, whether these cases are associated with recent *Mycobacterium tuberculosis* transmission or represent reactivation of past disease. During 2009–2015, we performed a population-based TB investigation in Yamagata Prefecture, Japan, using in-depth contact tracing and 24-loci variable-number tandem-repeat typing optimized for Beijing family *M. tuberculosis* strains. We analyzed 494 strains, of which 387 (78.3%) were derived from elderly patients. Recent transmission with an epidemiologic link was confirmed in 22 clusters (70 cases). In 17 (77.3%) clusters, the source patient was elderly; 11 (64.7%) of the 17 clusters occurred in a hospital or nursing home. In this setting, the increase in TB cases was associated with *M. tuberculosis* transmissions from elderly persons. Prevention of transmission in places where elderly persons gather will be an effective strategy for decreasing TB incidence among predominantly elderly populations.

The World Health Organization End TB strategy (*1*) calls for every country, depending on their tuberculosis (TB) situation, to accelerate efforts designed to end TB. In Japan, 14.4 TB cases/100,000 population were reported in 2015. A small percentage of those cases occurred in foreign-born (6.4%) and HIV-positive (<0.1%) persons, who thus are not currently considered to pose a transmission threat; however, 71.8% of the reported cases were in elderly persons (≥60 years of age) (*1*,*2*). Given that the incidence of TB was high in Japan until the 1970s (*3*), the current elderly population is regarded as vulnerable to TB onset from reactivation of remotely acquired latent infection (*4*,*5*). However, TB transmission among elderly populations has not been determined worldwide (*6*).

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Molecular epidemiology can help identify recent TB transmission and, thereby, contribute to the creation of specific intervention programs for advanced TB control. Variable-number tandem-repeat (VNTR) typing is a useful method for rapidly detecting TB infections caused by the same strain of *Mycobacterium tuberculosis* (*7*); such cases might occur from recent transmission. A combination of VNTR typing and in-depth contact tracing has detected previously unrecognized recent TB transmission in various settings (*8*–*11*). However, data are scarce from areas such as Japan that might include large numbers of patients with reactivated TB (i.e., reactivation of past latent TB infection because of a weakened immune system).

The Beijing family, within lineage 2 (East Asian) of *M. tuberculosis*, has shown cumulative microevolution while spreading globally from its origin in eastern Asia (*12*–*16*). Worldwide, the modern Beijing subfamily predominates (*16*), but in Japan, the ancient Beijing subfamily, which is subdivided into 4 sublineages (ST11/26, STK, ST3, and ST25/19), accounts for >75% of Beijing family strains (*17*–*19*). Thus, appropriate VNTR subsets were proposed to discriminate Beijing *M. tuberculosis* lineages (*20*,*21*); a 24-loci subset, named 24_{Beijing}, is used to achieve high discriminatory power for *M. tuberculosis* Beijing clinical isolates in Japan (*19*,*21*). The subset comprises 15-loci mycobacterial interspersed repetitive units (MIRUs) (*7*) and 9 additional loci, including 3 hypervariable loci (*21*).

We performed a population-based TB investigation in Yamagata Prefecture, Japan, combining 24_{Beijing}-VNTR typing and in-depth contact tracing. We aimed to clarify the overall picture of recent *M. tuberculosis* transmission and thereby contribute to preventing the spread of TB in Yamagata Prefecture, where the annual TB incidence during the past decade was 7–13 cases/100,000 persons (*3*) and where 80.5% of reported patients were ≥60 years of age in 2015 (*2*). The investigation was designed to provide insight about TB among predominantly elderly populations as an aid to countries experiencing an increase in TB cases among the elderly (*1*,*22*,*23*). This work was approved by

the Ethics Committees of Yamagata Prefectural Institute of Public Health (approval no. YPIPHEC H24–04 and YPIPHEC 16–08) and of the Institute of Tropical Medicine, Nagasaki University (approval no. 130606112).

Materials and Methods

Study Setting

Yamagata Prefecture, located in the northern part of Japan's main island of Honshu, is subdivided into 4 topographically separated areas, each of which has a public health center (online Technical Appendix 1 Figure 1, https://wwwnc.cdc.gov/EID/article/23/3/16-1571-Techapp1.pdf). In 2014, Yamagata Prefecture had 1.1 million inhabitants, of whom 38.2% were elderly (≥60 years of age) and 0.5% were noncitizen residents. Statistical data used in the study was provided by the Statistics Planning Division, Yamagata Prefecture.

Study Population

The study included most patients in Yamagata Prefecture with culture-confirmed TB reported during January 1, 2009–December 31, 2015. On the basis of Japan's Law Regarding Infectious Disease Prevention and Medical Care for the Patients, which was implemented on April 1, 1999, public health centers collect *M. tuberculosis* strains isolated from patients in order to conduct molecular investigations. During 2009–2015, public health centers routinely delivered collected strains to the Yamagata Prefectural Institute of Public Health within 2 months after notification of TB cases. These collected strains corresponded in part to those described in our earlier works, which mainly analyzed genetic features of *M. tuberculosis* (e.g., phylogenetic classification and the genome sequence) (*19*,*24*,*25*).

Genotyping

We usually finished $24_{Beijing}$-VNTR typing within 3 days after arrival of the strains. We confirmed the amplified PCR fragment sizes by using a microchip electrophoresis system (MCE–202; Shimadzu Corp., Kyoto, Japan) and agarose gel electrophoresis. We calculated the number of repeats for each locus from the sizes of PCR products, in agreement with published allelic tables (*26*). For this study, we defined a preliminary TB cluster when the $24_{Beijing}$-VNTR profile of a strain was a single-locus variant (SLV) or was indistinguishable from that of other strains.

We estimated *M. tuberculosis* lineages of the clinical isolates by using maximum a posteriori estimation with the $24_{Beijing}$-VNTR profile, as described previously (*19*). We divided the strains into 6 lineages: the group of non-Beijing *M. tuberculosis* lineages, 4 sublineages (ST11/26, STK, ST3, and ST25/19) of ancient Beijing subfamily, and the modern Beijing subfamily.

Data Collection

Public health centers routinely collect demographic (age, sex, country of birth, and address), clinical (site of disease, acid-fast bacilli sputum smear status, and treatment history), epidemiologic (family members, occupation, and contacts during onset), and microbiologic (culture and drug-susceptibility status of *M. tuberculosis* strain) characteristics for all reported TB patients. Public health centers use these data to determine whether interferon-γ release assays and chest radiography should be used to determine whether contacts of patients have latent TB infection. When a preliminary TB cluster is confirmed, public health nurses investigate the behavior history of patients within clusters to determine recent *M. tuberculosis* transmission. If patients in a cluster live in dispersed areas, the investigations are performed in cooperation with the responsible public health centers. In addition, after November 2013, public health centers asked all patients with culture-confirmed TB to complete a long or short version of a self-administered questionnaire that specifically elicits responses associated with residence, travel history, transportation, and places of social aggregation (online Technical Appendix 2, https://wwwnc.cdc.gov/EID/article/23/3/16-1571-Techapp2.pdf). Public health centers decide which version of the questionnaire to use, depending on patient willingness and ability to fill out the form. For example, in this study, the short version was used for elderly TB patients (especially those ≥80 years of age) who exhibited forgetfulness, tremulousness of hands, or an unwillingness to complete the questionnaire. Using social interaction data gathered from the case investigations and questionnaires, public health centers graded patients within clusters as epidemiologically linked (i.e., patients had shared space at the same time); possibly linked (i.e., patients had shared space but not at the same time); or not linked (i.e., no shared space was found for patients), according to the classification method of Walker et al. (*27*).

Cluster Analysis

We defined cases as a cluster when their $24_{Beijing}$-VNTR profiles were indistinguishable from each other or when a social interaction was graded as epidemiologically linked or possibly linked in the SLV group. We excluded nonlinked cases in the SLV group from the cluster, based on the assertion by Allix-Béguec et al. that "even for hypervariable loci, at least in the absence of further specific epidemiological or contact tracing evidence, the definition of molecular clustering should remain restricted to full identity of the markers" (*28*). By comparing 24-loci MIRU-VNTR profiles proposed by Supply et al. (*7*) and results of whole-genome sequencing, Walker et al. (*27*) showed that *M. tuberculosis* strains in the SLV group contained many more single-nucleotide polymorphisms than those in the indistinguishable group.

We calculated the proportion of clustered cases resulting from recent *M. tuberculosis* transmission by using the $n - 1$ method, defined as $(N_c - n_c)/N_o$, where N_c stands for the total number of clustered cases, n_c signifies the number of clusters (i.e., equal to the number of source cases), and N_o denotes the total number of cases in this study (*9*,*29*). In addition, we calculated the percentage of cases resulting from epidemiologic links, including only cases and clusters with confirmed links in the calculation [(no. of clustered cases with epidemiologic links – no. of clusters with epidemiologic links)/N_o] (*9*). We visualized cluster diagrams displaying epidemiologic links by using Cytoscape 3.3.0, an open-source bioinformatics software platform (*30*).

Statistical Analysis

We calculated odds ratios and 95% CIs by using logistic regression analysis. To determine the association between cluster formation and epidemiologic features (especially age groups), we applied multivariate logistic regression analysis using, for example, age group, sex, and *M. tuberculosis* lineage as explanatory variables. We used backward, stepwise variable selection to select the multivariate model with a probability entry of <0.2. Residual analysis was used when logistic regression analysis was not applicable. We considered $p<0.05$ as statistically significant. All statistical analyses were conducted using R version 3.0.2 (R Foundation for Statistical Computing, Vienna, Austria).

Results

During 2009–2015, a total of 854 TB cases were reported in Yamagata Prefecture; 676 of the cases were diagnosed as pulmonary TB, of which 513 were culture-confirmed. We studied 494 (57.8%) of the 854 cases (469 [91.4%] of the 513 pulmonary cases and 25 extrapulmonary cases). We collected *M. tuberculosis* strains from the patients and determined the $24_{Beijing}$-VNTR profiles. Patients had a mean (±SD) age of 72.3 ± 19.9 (range 18–100) years. Most patients were ≥60 years of age (387 patients, 78.3%), Japanese (478 patients, 96.8%), and undergoing initial TB treatment (466 patients, 94.3%), and most had pulmonary symptoms (469 patients, 94.9%) and non–multidrug-resistant TB (491 patients, 99.4%). No patients were HIV-positive, illicit drug users, or homeless.

Results of $24_{Beijing}$-VNTR typing showed that 173 strains formed 52 preliminary clusters, which were aggregations of indistinguishable and SLV profiles (online Technical Appendix 1 Table 1). Of note, the proportion of nonlinked cases in the SLV group was remarkably high (45/57, 78.9%) when we separated cases belonging to the preliminary cluster into indistinguishable and SLV groups (Table 1). Considering these findings and the assertion of Allix-Béguec et al. (*28*), we excluded 45 cases for which $24_{Beijing}$-VNTR profiles formed SLV clusters without epidemiologic links. The remaining 128 cases formed 42 clusters (online Technical Appendix 1 Table 1), each of which contained 2–17 cases; 45.3% (58/128) of cases were in small clusters (2 cases), 25.0% (32/128) were in medium clusters (3 or 4 cases), and 29.7% (38/128) were in 3 large clusters (7, 14, and 17 cases, respectively). Cases attributable to recent transmission accounted for 17.4% (128 clustered cases – 42 clusters/494 total cases).

In both univariate and multivariate analyses, odds ratios for cluster formation were lower for patients ≥60 years of age than those ≤39 of age (Table 2). However, among the *M. tuberculosis* lineages, only ancient Beijing lineage ST11/26, which was represented by cluster 12, the largest cluster (n = 17; online Technical Appendix 1 Table 1), showed a markedly high odds ratio against the modern Beijing subfamily in both univariate and multivariate analyses. The odds ratios of ancient Beijing lineages STK and ST3 against the modern Beijing lineage were significant only with univariate analysis. We also determined the risk for infection with the different *M. tuberculosis* lineages by age group (Table 3). The proportion of infections with STK, ST3, and ST25/19 was remarkably high among patients ≥60 years of age, whereas the proportion of infections with the modern Beijing subfamily was significantly higher ($p<0.01$) among patients ≤59 years of age.

We confirmed epidemiologic links in 22 (52.4%) of the 42 TB clusters that occurred during 2009–2015; the linked cases consisted of 20 source cases (i.e., the source of *M. tuberculosis* transmission) and 50 secondary TB cases (Figure) (online Technical Appendix 1 Figure 2). For each cluster, we identified a source case by information about the patients (e.g., the time of diagnosis, degree of infectiousness, the start of the infectious period based on sputum smear status, severity and duration of respiratory symptoms, findings on chest radiographs, and sociability of the patient). Source cases in clusters 12 and 34 were not included in this study because they occurred outside the study period or lacked a VNTR profile. The most common transmission setting was hospitals that had cared for TB

Table 1. Crude odds ratio for single-locus variant among 173 *Mycobacterium tuberculosis* strains forming preliminary clusters, by epidemiologic links of tuberculosis cases, Yamagata Prefecture, Japan, 2009–2015

Epidemiologic link	$24_{Beijing}$-VNTR profile, no. (%)		Crude odds ratio (95% CI)*
	Indistinguishable, n = 116	Single-locus variant, n = 57	
Linked	42 (36.2)	11 (19.3)	1.0
Possibly linked	16 (13.8)	1 (1.8)	0.2 (0.03–2.0)
Not linked	58 (50.0)	45 (78.9)	**3.0 (1.4–6.4)**

*CIs that do not overlap the null value of odds ratio = 1 are shown in bold.

Table 2. Odds ratio for cluster formation among 494 persons with tuberculosis, Yamagata Prefecture, Japan, 2009–2015*

Patient characteristic	$24_{Beijing}$-VNTR profile, no. (%)		Odds ratio (95% CI)†	
	Not clustered, n = 366	Clustered, n = 128	Univariate	Multivariate‡
Age group				
≤39	31 (8.5)	30 (23.4)	1.0	1.0
40–59	25 (6.8)	21 (16.4)	0.9 (0.4–1.9)	0.8 (0.3–1.8)
60–79	96 (26.2)	35 (27.3)	**0.4 (0.2–0.7)**	**0.4 (0.2–0.8)**
≥80	214 (58.5)	42 (32.8)	**0.2 (0.1–0.4)**	**0.2 (0.1–0.4)**
Sex				
F	143 (39.1)	59 (46.1)	1.3 (0.9–2.0)	1.6 (0.99–2.4)
M	223 (60.9)	69 (53.9)	1.0	1.0
Birthplace				
Japan	351 (95.9)	127 (99.2)	5.4 (0.7–41.5)	**16.7 (2.0–137.0)**
Other	15 (4.1)	1 (0.8)	1.0	1.0
Site of disease				
Pulmonary, sputum smear–positive	251 (68.6)	78 (60.9)	1.0	–
Pulmonary, sputum smear–negative	97 (26.5)	44 (34.4)	1.5 (0.9–2.3)	–
Extrapulmonary	18 (4.9)	6 (4.7)	1.1 (0.4–2.8)	–
Treatment history				
Initial	343 (93.7)	123 (96.1)	1.6 (0.6–4.4)	–
Retreatment	23 (6.3)	5 (3.9)	1.0	–
M. tuberculosis lineage				
Non-Beijing	102 (27.9)	38 (29.7)	0.7 (0.4–1.4)	1.2 (0.6–2.3)
ST11/26	14 (3.8)	19 (14.8)	**2.7 (1.2–6.3)**	**2.5 (1.02–6.1)**
STK	72 (19.7)	10 (7.8)	**0.3 (0.1–0.6)**	0.4 (0.2–1.1)
ST3	68 (18.6)	15 (11.7)	**0.4 (0.2–0.9)**	0.8 (0.3–1.7)
ST25/19	60 (16.4)	21 (16.4)	0.7 (0.4–1.4)	1.0 (0.5–2.2)
Modern Beijing	50 (13.7)	25 (19.5)	1.0	1.0

*–, no variables.
†CIs that do not overlap the null value of odds ratio = 1 are shown in bold.
‡Adjusted for the other factors used in the multivariate model.

patients (15 [30.0%] of 50 secondary cases), followed by households (14 [28.0%] of 50 secondary cases). We found unsuspected links for 23 (46.0%) of 50 secondary cases by conducting in-depth contact tracings after VNTR typing. Among the 23 cases, 22 (95.7%) aggregated with other TB cases in settings outside the household: hospitals (13 cases), pachinko parlors (7 cases), a nursing home (1 case), and a sporting event (1 case). The proportion of cases attributable to recent transmission after adjustment for epidemiologic links was 9.7% (70 clustered cases with epidemiologic links – 22 clusters with epidemiologic links/494 total cases). Furthermore, because unsuspected transmission settings were found within clusters 03, 12, and 34 after VNTR typing, we performed interferon-γ release assays on samples from close contacts of TB case-patients in those settings; none of the results were positive (data not shown).

For the large clusters (clusters 12, 26, and 34), we confirmed that there had been a delay between symptom onset and diagnosis for the source patients or that the source patients were highly socially active (online Technical Appendix 1 Table 2). In cluster 12, we studied 17 cases reported during 2009–2015; another 18 cases were excluded from the study because they occurred outside the study period (online Technical Appendix 1 Figure 2). In cluster 34, a diagnosis of TB in the probable source case-patient was missed because the patient had been diagnosed with lung cancer. However, a typical tuberculous cavity was found by retrospective viewing of a chest radiograph taken before the patient's death.

Seventeen clusters originating from elderly patients were of small or medium size; the exception was cluster 34, for which the source case had not been diagnosed as TB (online Technical Appendix 1 Figure 2). Source cases of the clusters aggregated with secondary cases in hospitals (9 clusters), households (7 clusters), and nursing homes (2 clusters). Almost all of the secondary case-patients aggregating at households and nursing homes were elderly persons, whereas most secondary case-patients within the

Table 3. Lineages of 494 *Mycobacterium tuberculosis* strains by patient age group in Yamagata Prefecture, Japan, 2009–2015

Patient age group, y	No. (%) patients	*M. tuberculosis* lineage, no. (%)					
			Ancient Beijing				Modern Beijing
		Non-Beijing	ST11/26	STK	ST3	ST25/19	
≤39	61 (100)	16 (26.2)	13 (21.3)*	4 (6.6)	2 (3.3)	3 (4.9)	23 (37.7)*
40–59	46 (100)	12 (26.1)	4 (8.7)	2 (4.3)	1 (2.2)	10 (21.7)	17 (37.0)*
60–79	131 (100)	37 (28.2)	6 (4.6)	19 (14.5)	24 (18.3)	30 (22.9)†	15 (11.5)
≥80	256 (100)	75 (29.3)	10 (3.9)	57 (22.3)*	56 (21.9)*	38 (14.8)	20 (7.8)

*Significantly higher proportion by residual analysis ($p<0.01$).
†Significantly higher proportion by residual analysis ($p<0.05$).

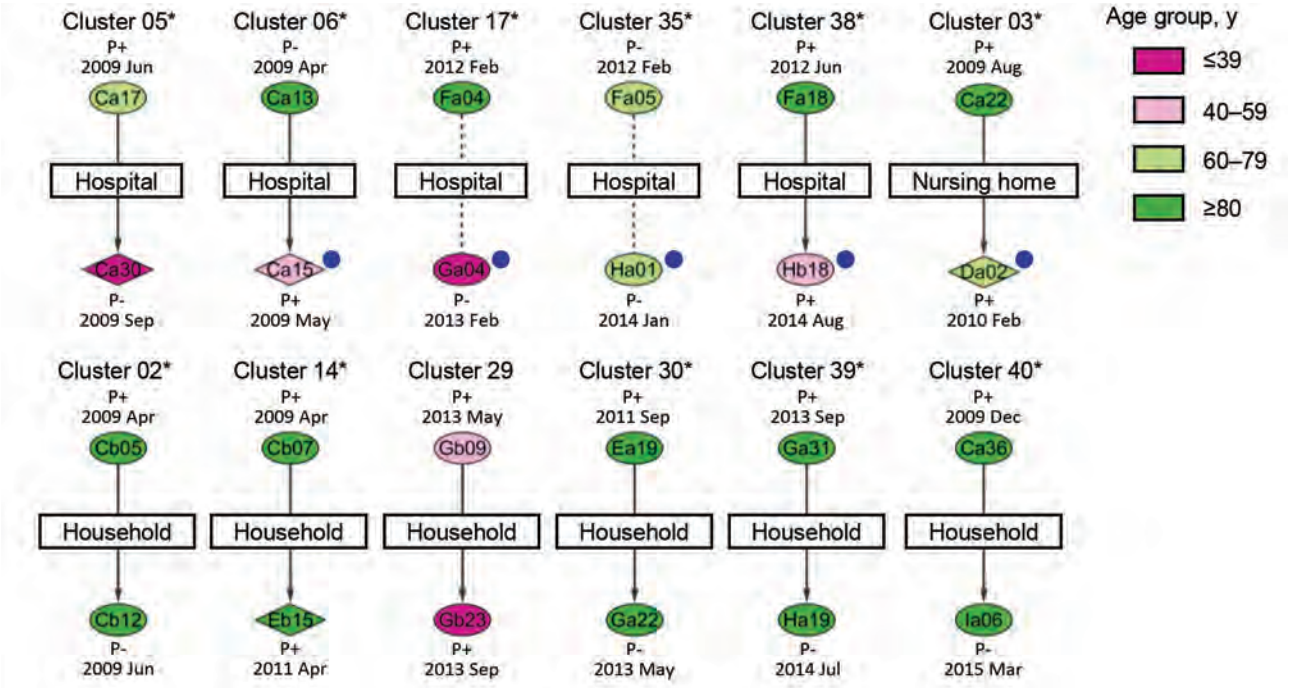

Figure. Twelve small tuberculosis (TB) clusters (2 cases each) among a total of 22 clusters with epidemiologic links between patients, Yamagata Prefecture, Japan, 2009–2015. Ovals and diamonds represent individual cases, by patient age group, in each cluster; numbers inside symbols are patient identification codes. Ovals indicate cases with an indistinguishable 24-loci variable-number tandem-repeat typing profile optimized for Beijing family *M. tuberculosis* strains ($24_{Beijing}$-VNTR profile); diamonds indicate cases with a single-locus variant profile. Vertical arrows and dotted lines between cases within a cluster indicate linked and possibly linked cases, respectively. Transmission settings for linked cases are shown within rectangles. Case notification dates and patient disease sites are shown above/below the case symbol; P+ and P– indicate pulmonary smear–positive and –negative cases, respectively. Black dots indicate confirmation of the epidemiologic link by in-depth contact tracings after $24_{Beijing}$-VNTR typing. Asterisks indicate clusters that began with a TB source patient who was ≥60 years of age. An expanded version of this figure is available as online Technical Appendix 1 Figure 2 (https://wwwnc.cdc.gov/EID/article/23/3/16-1571-Techapp1.pdf).

hospital clusters were younger persons. Twelve (80.0%) of 15 secondary cases in hospital settings were in women ≤59 years of age, including 11 patients who were working as nurses or nurse's aides.

To investigate risk factors for nonlinked results of contact tracings, we analyzed characteristics of 128 clustered cases (Table 4). Multivariate analysis showed that elderly persons and *M. tuberculosis* lineages (non-Beijing and ancient Beijing) were independent risk factors for nonlinked results of contact tracings.

Discussion

We conducted molecular and epidemiologic TB investigations among a population in which 78.3% of the patients were ≥60 years of age. Among 22 TB clusters with epidemiologic links, 17 (77.3%) were traced to an elderly source patient (online Technical Appendix 1 Figure 2). Of those 17 clusters, 11 occurred in hospitals and nursing homes. Transmission in these settings involved secondary cases mostly among younger persons. Our findings indicate that elderly persons must be included in TB prevention and control measures in countries with low to moderate incidence that have experienced a shifting of TB cases toward the elderly (*1*,*22*,*23*).

Our results suggest that elderly patients with TB are a source of TB spread involving younger persons. Borgdorff et al. showed that the number of secondary cases generated per source case decreased concomitantly with increasing age of the source patient; source cases involving elderly persons tend to form clusters of TB cases among elderly persons (*6*). Our findings show that 16 of 17 clusters that began with an elderly source patient were small or medium size; however, cluster 34, caused by an elderly person who was not diagnosed with TB, formed a large cluster. Eleven clusters that had an elderly source patient included transmissions in hospitals and nursing homes, and 80% of secondary cases in hospital settings were in persons ≤59 years of age. Elderly patients with TB often lack typical symptoms, such as cough and fever (*31*,*32*). In addition, diagnosis of TB in elderly persons might be delayed because they sometimes have multiple underlying diseases such as aspiration pneumonia and chronic obstructive pulmonary disease. Given that various factors may delay TB diagnosis in elderly persons, efforts should be taken to decrease

Table 4. Risk factors for nonlinked results of contact tracings among 128 TB cases clustered in Yamagata Prefecture, Japan, 2009–2015

Characteristic	No. (%) cases with epidemiologic link		Odds ratio (95% CI)*	
	Linked or possibly linked	Not linked	Univariate	Multivariate†
Patient age group				
≤59	37 (52.9)	14 (24.1)	1.0	1.0
≥60	33 (47.1)	44 (75.9)	**3.5 (1.6–7.6)**	**2.9 (1.3–6.6)**
Patient sex				
F	39 (55.7)	20 (34.5)	1.0	1.0
M	31 (44.3)	38 (65.5)	**2.4 (1.2–4.9)**	2.2 (0.994–4.9)
Mycobacterium tuberculosis lineage				
Non-Beijing	16 (22.9)	22 (37.9)	**15.8 (3.3–76.9)**	**12.5 (2.5–63.3)**
Ancient Beijing	31 (44.3)	34 (58.6)	**12.6 (2.7–57.9)**	**10.1 (2.1–48.2)**
Modern Beijing	23 (32.9)	2 (3.4)	1.0	1.0

*CIs that do not overlap the null value of odds ratio = 1 are shown in bold.
†Adjusted for the other factors used in the multivariate model.

such delays in order to stop or control the spread of TB. Furthermore, information regarding TB prevention should be provided to workers who have close and routine contact with elderly persons (e.g., hospital and nursing home staff).

Contact investigations are necessary to acquire the greatest public health benefit for most infectious diseases. Our findings confirmed unsuspected links in 46.0% of secondary TB cases through in-depth contact tracing after VNTR typing (online Technical Appendix 1 Figure 2). In addition, we sought undiscovered latent TB infection cases by using interferon-γ release assays to find patient contacts in unsuspected settings. Given that earlier studies performed similar investigations and found latent and active TB cases (*8*,*9*,*11*), a combination of population-based molecular typing and further contact investigation is expected to be an effective strategy for discovering unknown latent TB infections or active TB cases. Moreover, we examined links in detail for 57 patients infected with *M. tuberculosis* strains in the SLV group; our findings confirmed epidemiologic links in 21.1% (12/57) of the cases (Table 1). These results indicate that in-depth contact tracings should be performed for TB cases caused by *M. tuberculosis* strains with SLV profiles.

The results of our study suggest that clusters that include elderly persons without epidemiologic links might be caused by the past endemic *M. tuberculosis* strains. We estimated, on the basis of molecular typing (*9*), that 17.4% (128 clustered cases – 42 clusters/494 total cases) of TB cases in this study were attributable to recent transmission; however, after we made adjustment for epidemiologic links, only 9.7% (70 clustered cases with epidemiologic links – 22 clusters with epidemiologic links/494 total cases) of the cases were attributable to recent transmission. The main cause for this difference in case numbers is that epidemiologic links were difficult to confirm for clustered cases among elderly persons (Table 4). In Japan, cases of TB among the elderly are attributable mainly to the endogenous relapse of *M. tuberculosis* infections that occurred near the time of World War II, when TB was highly prevalent (*4*,*5*). This fact suggests that past endemic strains, having indistinguishable VNTR profiles, have been isolated from elderly persons who have onset of TB in modern times caused by reactivation of latent TB infection. The confirmation of settings with recent *M. tuberculosis* transmission can guide interventions to control the spread of TB; however, the lack of such confirmation, despite in-depth contact tracings, may suggest infection with the past endemic *M. tuberculosis* strain, and the transmission settings for such infections can be difficult to detect.

Epidemiologic data together with phylogenetic information for *M. tuberculosis* strains causing infections might be useful in determining the time of TB infection. In this study, more than half of the strains were of the ancient Beijing subfamily, and the proportion of several sublineages from this family were markedly high in persons ≥60 years of age, whereas modern Beijing subfamily strains were prevalent in younger age groups (Table 3). Given that an earlier Japanese study found a similar tendency (*17*), predominant lineages will have shifted over time, at least in Japan. Such historical dynamics might provide valuable clues for estimating the background of isolated strains. For example, if nonclustered strains with STK or ST3 were isolated from elderly patients in Japan, then it might be reasonable that such cases were regarded as sporadic because of reactivation.

Our study had several potential limitations. First, contact tracings might not clarify all epidemiologic links among clustered TB cases. In particular, findings of the true location of transmission for young and active persons with TB may be missed because those persons tended to frequent many locations. By contrast, elderly persons with TB frequented fewer locations, but their disabilities (e.g., forgetfulness and impaired hearing) or death immediately after TB diagnosis compromised our data gathering. To overcome this limitation, the use of whole-genome sequencing, which has higher discriminatory power than VNTR typing, might indicate whether clustered strains are derived from recent transmission (*27*,*33*). Second, *M. tuberculosis*

genotyping studies cannot investigate culture-negative TB cases. This unavoidable limitation caused a decrease of overall coverage in our study (i.e., >40% of TB cases were beyond the scope of investigation). However, our comprehensive investigation of culture-confirmed TB cases, which are more infectious than culture-negative cases (*34*) and form the basis of TB transmission, may have been sufficient for determining the representative transmission settings in our study area. Our findings from Yamagata Prefecture provide empiric evidence that nonhousehold settings populated or frequented by elderly persons (e.g., hospitals and social gathering settings) are hotspots for *M. tuberculosis* transmission among this population. The last limitation is that our study was restricted to a local setting. However, Theron et al. (*35*) recently proposed that an important factor for ending TB epidemics is to emphasize strategies at the local level, where TB transmission occurs. The accumulation of empiric evidence for various other local settings in Japan is expected to become a higher priority for decision-making for nationwide policies regarding TB.

In summary, molecular genotyping methods make it possible to perform evidence-based TB control (*8–11*,*27*). We confirmed the effectiveness of these methods in a mostly elderly population by using VNTR typing with in-depth contact tracing. Our results suggest that prevention of *M. tuberculosis* transmissions in places where elderly persons gather can be an effective strategy for decreasing TB incidence. A combination of molecular and epidemiologic data can assist public health officials in obtaining an overview of recent transmission and in detecting unsuspected transmission settings, thereby enabling further informational activities and interventions to prevent the spread of TB.

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Comparison of Sputum-Culture Conversion for *Mycobacterium bovis* and *M. tuberculosis*

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Current US guidelines recommend longer treatment for tuberculosis (TB) caused by pyrazinamide-resistant organisms (e.g., *Mycobacterium bovis*) than for *M. tuberculosis* TB. We compared treatment response times for patients with *M. bovis* TB and *M. tuberculosis* TB reported in the United States during 2006–2013. We included culture-positive, pulmonary TB patients with genotyping results who received standard 4-drug treatment at the time of diagnosis. Time to sputum-culture conversion was defined as time between treatment start date and date of first consistently culture-negative sputum. We analyzed 297 case-patients with *M. bovis* TB and 30,848 case-patients with *M. tuberculosis* TB. After 2 months of treatment, 71% of *M. bovis* and 65% of *M. tuberculosis* TB patients showed conversion of sputum cultures to negative. Likelihood of culture conversion was higher for *M. bovis* than for *M. tuberculosis*, even after controlling for treatment administration type, sex, and a composite indicator of bacillary burden.

The *Mycobacterium tuberculosis* complex is composed of several genetically related and pathogenic mycobacterial species, including *M. tuberculosis* and *M. bovis* (*1*). Tuberculosis (TB) caused by these species is often clinically indistinguishable, although *M. bovis* has a different epidemiologic profile (*2*–*5*). Despite similarities, there is growing evidence that diversity within the *M. tuberculosis* complex has major immunologic consequences, which may influence treatment response (*6*–*11*). Sputum-culture conversion (i.e., conversion from positive to negative culture result) is considered the principal prognostic indicator for treatment response and is often used as a surrogate endpoint in early-phase randomized clinical trials (*12*,*13*). Studies have demonstrated that sputum-culture conversion differs by *M. tuberculosis* phylogenetic lineage (*14*,*15*).

M. bovis is generally considered intrinsically resistant to pyrazinamide, which is considered an essential first-line anti-TB drug. Pyrazinamide is a sterilizing drug that acts synergistically with rifampin to shorten the duration of anti-TB treatment from 9 to 6 months (*16*). In the absence of this benefit, many experts currently recommend extending treatment for TB caused by *M. bovis* (*17*). However, these recommendations are based on expert opinion and lack definitive evidence from laboratory studies or randomized clinical trials (*17*). We evaluated differences in time from treatment initiation to sputum-culture conversion between patients with *M. bovis* TB and *M. tuberculosis* TB given standard first-line anti-TB treatment.

Methods

We analyzed data from the National Tuberculosis Surveillance System (NTSS) at the Centers for Disease Control and Prevention (Atlanta, GA, USA) and restricted analysis to cases reported during 2006–2013 to permit sufficient time for follow-up reporting of outcome data. *M. tuberculosis* complex isolates were identified by using spoligotyping and multilocus variable number tandem repeat (i.e., mycobacterial interspersed repetitive unit–variable number tandem repeat) genotyping techniques (*3*,*18*).

We used a retrospective cohort study design and included culture-confirmed TB cases with complete genotyping results and pulmonary disease treated with a standard 4-drug regimen (i.e., isoniazid, rifampin, ethambutol, and pyrazinamide) at diagnosis (*19*). Cases with isolates identified as any species other than *M. tuberculosis* or *M. bovis* were excluded; cases with isolates identified as *M. bovis* Bacillus Calmette–Guérin were assumed to be iatrogenic (*20*) and were also excluded. We excluded from analysis any case-patients with organisms initially resistant to rifampin or isoniazid, those infected with *M. tuberculosis* initially resistant to pyrazinamide, those who were dead at time of diagnosis, and those with missing or unreliable culture-conversion data. We used the Pearson χ^2 test to compare clinical and demographic characteristics of case-patients with *M. bovis* and *M. tuberculosis* TB and the proportion of case-patients who showed conversion of cultures at 2 and 3 months.

Time to sputum-culture conversion was calculated for persons with positive sputum cultures as the number

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of days from the date treatment started until the date of the first consistently culture-negative sputum. The date of the first consistently culture-negative sputum was defined as the date that a specimen was collected for the first documented negative culture result with no concurrent (i.e., samples collected within 1 week) or subsequent positive cultures. We used a Kaplan-Meier estimator to calculate survival-like function curves for time to sputum-culture conversion for persons with *M. bovis* and *M. tuberculosis* TB.

We censored, at the date recorded for each outcome, patients who died during treatment and those who moved, were lost to follow-up, or otherwise stopped treatment before the expected completion date; we restricted the analysis to the first 90 days of anti-TB treatment. We used Cox proportional hazard modeling to calculate adjusted hazard ratios (aHRs) with 95% CIs of factors associated with time to culture conversion. We tested the proportional hazards assumption by graphing log (–log [survival probability]) versus log (time) for all covariates of interest. We assessed a priori covariates of interest, including sex; treatment administration type (directly observed therapy versus self-administered therapy); reported HIV status (positive, negative, and unknown); sputum smear status (positive, negative, not obtained, and unknown); and cavitary disease found by diagnostic imaging (chest radiography or computed tomography; positive, negative, not done, and unknown) results.

After reviewing smear status and diagnostic imaging results, we created a composite variable for bacillary burden (high, medium, low, and unknown) to avoid collinearity. We categorized cases with confirmed cavitary disease identified by imaging and positive sputum smear as high bacillary burden, cases with either confirmed cavitary disease or positive sputum smear as medium bacillary burden, cases with no cavitary disease and negative sputum smears as low bacillary burden, and cases without any of these indicators as unknown bacillary burden. In the time-to-event analysis, we excluded patients who had unknown bacillary burden. Analysis was conducted by using SAS version 9.3 (SAS Institute, Cary, NC, USA).

Results

A total of 91,985 TB cases during 2006–2013 were available for analysis (Figure 1). Approximately two thirds (64.4%) of these cases had complete genotyping results and were identified as either *M. tuberculosis* TB or *M. bovis* TB. All cases of TB with initial resistance to isoniazid or rifampin (n = 4,418) and *M. tuberculosis* TB cases with any initial resistance to pyrazinamide (n = 757) were excluded. The final dataset for analysis included 297 cases of *M. bovis* TB and 30,848 cases of *M. tuberculosis* TB. All covariates met the proportional hazards assumption except for HIV status, which was excluded from further analysis because of sparse data.

The age distributions of patients given a diagnosis of *M. bovis* TB and patients given a diagnosis of *M. tuberculosis* TB were similar (Table 1). A greater proportion of patients with *M. bovis* TB were female (123/297 [41.1%]) than patients with *M. tuberculosis* TB (10,536/30,848 [34.2%]; p = 0.01). Patients with *M. bovis* TB were most often born outside the United States (86.2%), and most

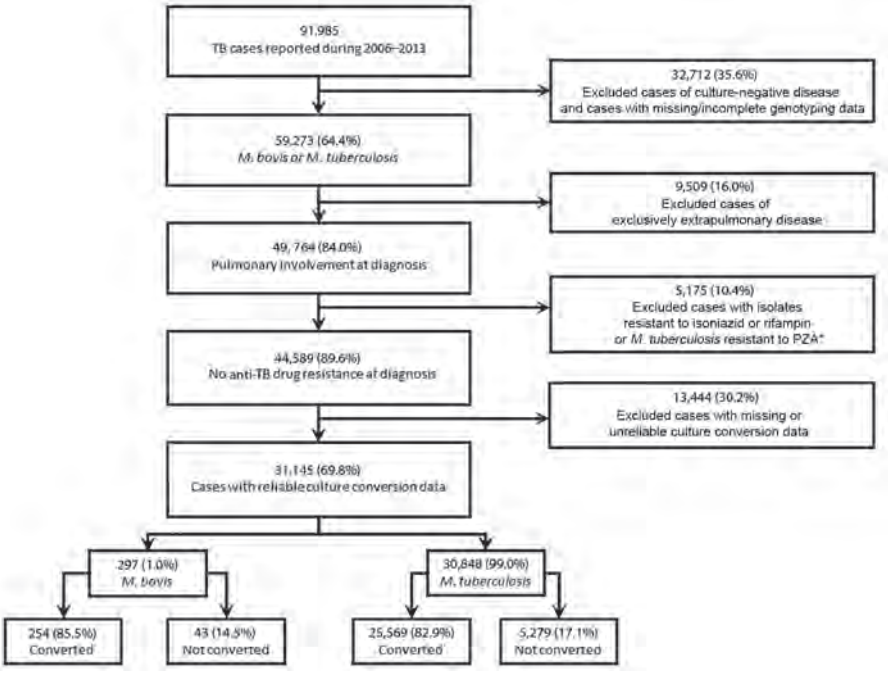

Figure 1. Selection of tuberculosis cases for analysis of sputum-culture conversion, United States, 2006–2013. Analysis included cases of culture-positive disease. A total of 61% of case-patients with *M. bovis* TB and 63% of case-patients with *M. tuberculosis* TB met analytic requirements for inclusion. PZA, pyrazinamide; TB, tuberculosis. **Mycobacterium tuberculosis* with pyrazinamide resistance (n = 757).

self-identified as Hispanic (93.2%). In comparison, just over half (59.5%) of *M. tuberculosis* TB patients were born outside the United States; 30.3% self-identified as Hispanic (p<0.0001 for both comparisons).

Approximately one third (35.0%) of *M. bovis* TB patients had pulmonary and extrapulmonary involvement at diagnosis compared with 10.0% of *M. tuberculosis* TB patients (p<0.0001). Similar proportions of patients had cavitary chest lesions documented (*M. bovis* TB = 46.6%, *M. tuberculosis* TB = 45.0%; p = 0.64) and positive sputum smear results (*M. bovis* TB = 63.6%, *M. tuberculosis* TB = 67.3%; p = 0.40). Among *M. tuberculosis* TB patients, proportions categorized as high bacillary burden (36.2%) and medium bacillary burden (37.1%) differed significantly from those for *M. bovis* TB patients (28.3% and 40.4%, respectively) (p = 0.03).

At 2 months of treatment, 71% of *M. bovis* TB patients and 65% of *M. tuberculosis* TB patients showed conversion

Table 1. Characteristics of patients with pulmonary tuberculosis caused by *Mycobacterium bovis* or *Mycobacterium tuberculosis*, United States, 2006–2013*

Variable	Cause of tuberculosis		p value†
	M. bovis, n = 297	*M. tuberculosis*, n = 30,848	
Age, y			
0–4	0	18 (0.1)	0.52
5–14	2 (0.7)	202 (0.7)	
15–24	46 (15.5)	3966 (12.9)	
25–44	105 (35.4)	10,553 (34.2)	
45–64	99 (33.3)	10,237 (33.2)	
≥65	45 (15.2)	5872 (19.0)	
Median age, y (interquartile range)	43 (29–56)	46 (30–60)	0.12
Sex			
M	174 (58.6)	20,303 (65.8)	0.01
F	123 (41.4)	10,536 (34.2)	
Race/ethnicity			
White, non-Hispanic	9 (3.0)	5205 (16.8)	<0.0001
Native American, non-Hispanic	0	456 (1.5)	
Asian, non-Hispanic	3 (1.0)	7869 (26.0)	
Black, non-Hispanic	8 (2.7)	7578 (25.0)	
Hispanic	276 (93.2)	9177 (30.3)	
Country of birth			
United States	41 (13.8)	12,442 (40.3)	<0.0001
Other‡	256 (86.2)	18,371 (59.5)	
Reported HIV status§			
Positive	17 (5.7)	1851 (6.0)	<0.0001
Negative	166 (55.9)	21,585 (70.0)	
Unknown	114 (38.4)	7412 (24.0)	
Clinical presentation at diagnosis			
Pulmonary disease	193 (65.0)	27,757 (90.0)	<0.0001
Pulmonary and extrapulmonary disease	104 (35.0)	3090 (10.0)	
Computed tomography or other chest imaging findings¶			
Cavitary disease	90/193 (46.6)	12,476/27,757 (45.0)	0.64
Noncavitary disease	103/193 (53.4)	15,281/27,757 (55.0)	
Sputum smear result			
Positive	189 (63.6)	20,744 (67.3)	0.40
Negative	98 (33.0)	9403 (30.5)	
Not obtained	10 (3.4)	689 (2.2)	
Bacillary burden#			
High	84 (28.3)	11,154 (36.2)	0.03
Medium	120 (40.4)	11,445 (37.1)	
Low	85 (28.6)	7734 (25.1)	
Unknown	8 (2.7)	515 (1.7)	
Treatment outcome**			
Completed	235 (83.9)	25,535 (90.5)	0.001
Died	25 (8.9)	1447 (5.1)	
Other††	20 (7.4)	1238 (4.4)	

*Values are no. (%) unless otherwise indicated. Sums of counts across categories for a specific factor might be less than total counts in the column headings because of missing or incomplete data.
†Determined by using Pearson χ^2 test unless otherwise indicated.
‡Restricted to foreign-born persons.
§HIV status data are unknown for patients reported for California and Vermont during 2006–2011. Unknown also includes data from other states reporting unknown HIV status for tuberculosis patients.
¶Cavitary status was documented if reports of either conventional radiography or computed tomography indicated its presence.
#A bacillary burden composite variable was created by using smear status and radiographic evidence of cavitation.
**Outcome data are available for, and restricted to, patients reported during 2006–2013.
††Includes treatment stopped because of adverse events; patients were lost to follow-up, moved, refused treatment; and unknown reasons.

of their sputum cultures to negative (p<0.01) (Figure 2). By the end of 3 months of treatment, 86% of *M. bovis* TB patients and 83% of *M. tuberculosis* TB patients showed conversion of their sputum cultures to negative (Figure 2). On the basis of Cox proportional hazards regression modeling, we found that *M. bovis* TB patients had a higher hazard of conversion of sputum cultures to negative (aHR 1.18, 95% CI 1.04–1.33) relative to *M. tuberculosis* TB patients, after controlling for treatment administration type, sex, and the composite indicator for bacillary burden (Table 2). Directly observed therapy (aHR 1.12, 95% CI 1.09–1.15, relative to self-administered therapy) and female sex (aHR 1.15, 95% CI 1.12–1.18) were also found to increase the hazards of sputum-culture conversion. A graded response to bacillary burden was observed (low, aHR 1.68, 95% CI 1.63–1.74; medium, aHR 1.32, 95% CI 1.29–1.36) relative to high bacillary burden.

Discussion

For patients given a standard 4-drug regimen in the United States, we found that the hazard of culture conversion over the first 3 months of anti-TB treatment was higher for patients with *M. bovis* TB than for patients with *M. tuberculosis* TB after controlling for treatment administration type, sex, and a composite indicator of bacillary burden. This finding was not documented previously and is especially intriguing because *M. bovis* is inherently resistant to pyrazinamide, a first-line anti-TB drug used during the first 2 months of treatment and credited with reducing relapse rates of the 6-month regimen to levels similar to the 9-month regimen without this drug (*17*). The implications of this inherent resistance are unknown. A systematic review of treatment for *M. bovis* TB concluded that the effect of 6-month versus 9-month treatment durations could not be determined because of a paucity of observational data (*21*). Our findings suggest that TB caused by *M. bovis* might not require a 9-month treatment regimen. However, this suggestion requires additional investigations.

We found that *M. bovis* TB patients had pulmonary and extrapulmonary involvement at diagnosis more frequently than *M. tuberculosis* TB patients, which is consistent with previous reports describing the transmission and epidemiology of *M. bovis* (*2*,*3*,*22*,*23*). However, our analysis showed that *M. bovis* TB patients with pulmonary disease might have been less infectious (i.e., had a lower bacillary burden) than *M. tuberculosis* TB patients. We also found similar proportions of cavitary lesions on chest imaging, which might indicate a similar pathogenicity between organisms when pulmonary disease is present. However, this finding could not be directly studied because NTSS does not collect data on smear grade. Alternatively, differential gene expression, proinflammatory macrophage response, growth in macrophages, and lipid profiles might have roles in explaining variability in bacillary populations between species (*7*–*11*). Further studies assessing differences in bacillary burden, and how anti-TB treatment is affected, will help clarify potential differences in treatment efficacy for *M. tuberculosis* complex species.

The true global burden of *M. bovis* TB is unknown and estimates are imprecise. Populations burdened by endemic zoonotic TB, such as large pastoralist populations who live near livestock and communities with increased consumption of unpasteurized dairy products, are often located where a specific *M. bovis* TB diagnosis is unlikely because extrapulmonary cases are not easily diagnosed and access to molecular technologies (e.g., genotyping) and drug susceptibility testing are not readily available (*24*–*27*). TB treatment strategies are moving toward shorter, more effective anti-TB regimens. If our finding of more rapid time to sputum-culture conversion for *M. bovis* TB

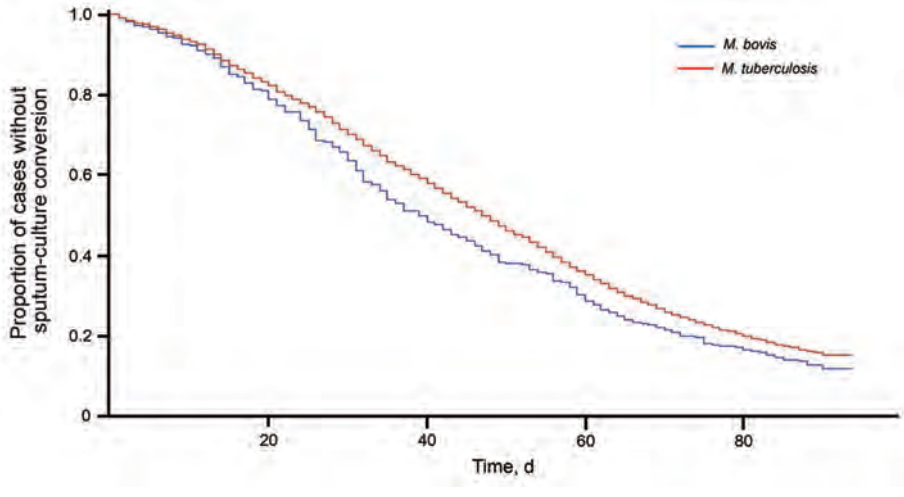

Figure 2. Time to sputum-culture conversion for case-patients with *Mycobacterium bovis* and *M. tuberculosis* TB, United States, 2006–2013. At day 0, a total of 297 persons had culture-positive *M. bovis* TB and 30,848 had culture-positive *M. tuberculosis* TB; at day 20, a total of 239 persons had culture-positive *M. bovis* TB and 25,363 had culture-positive *M. tuberculosis* TB; at day 40, a total of 143 persons had culture-positive *M. bovis* TB and 17,882 had culture-positive *M. tuberculosis* TB; at day 60, a total of 85 persons had culture-positive *M. bovis* TB and 10,853 had culture-positive *M. tuberculosis* TB; and at day 80, a total of 47 persons had culture-positive *M. bovis* TB and 6,084 had culture-positive *M. tuberculosis* TB. TB, tuberculosis.

Table 2. Hazard ratios for time to sputum-culture conversion among tuberculosis patients with pulmonary diagnoses, United States, 2006–2013*

Factor	Unadjusted HR (95% CI)	Adjusted HR (95% CI)
Cause of tuberculosis		
Mycobacterium bovis	1.18 (1.05–1.34)	1.18 (1.04–1.33)
M. tuberculosis	Referent	Referent
Treatment administration type		
Directly observed therapy	1.08 (1.05–1.11)	1.12 (1.09–1.15)
Self-administered	Referent	Referent
Sex		
F	1.17 (1.14–1.20)	1.15 (1.12–1.18)
M	Referent	Referent
Bacillary burden†		
Low	1.61 (1.56–1.66)	1.68 (1.63–1.74)
Medium	1.25 (1.22–1.29)	1.32 (1.29–1.36)
High	Referent	Referent

*HR, hazard ratio.
†A bacillary burden composite variable was created by using smear status and radiographic evidence of cavitation. Patients with unknown bacillary burden were excluded from analyses.

could be replicated with more robust randomized clinical trial studies or hollow-fiber models (*25*,*26*), recommendations for treatment might be improved. Modifying these standards could reduce the recommended treatment length for potentially hundreds of thousands patients globally, assuming a conservative 2%–3% *M. bovis* TB prevalence estimate among the 9.6 million cases of TB reported each year (*27*). As research elucidates mycobacterial features and characteristics, individualized treatment regimens might be tailored to specific organisms. However, many potential new regimens include pyrazinamide as an essential drug throughout the entire treatment course (*28*).

This study and its findings are subject to the limitations of the national surveillance data we used. Historically, there has been variation among states in the reporting of HIV testing results (*29*). California, which reports more than half of all *M. bovis* TB cases nationally each year, began reporting HIV test results to the Centers for Disease Control and Prevention in 2011. This reporting limited our ability to analyze any association between HIV and time to sputum-culture conversion for this study. Although there is a standard recommendation for follow-up sputum collection frequency after initiation of TB treatment, clinician and patient variability in implementation is likely. However, because there is no reason to believe that this variation would be implemented differently for *M. bovis* TB patients versus *M. tuberculosis* TB patients, this limitation is not likely to have affected comparison on the basis of species.

We attempted to control for potential variations in treatment by including only patients initially given a standard 4-drug regimen (isoniazid, rifampin, ethambutol, and pyrazinamide daily for 2 months). However, some clinicians might have changed regimens after receiving genotyping results (e.g., *M. bovis*) or drug susceptibility testing results (e.g., pyrazinamide resistance). Thus, some patients with *M. bovis* genotyping results or pyrazinamide resistance might have received a different regimen at some point after the start of treatment. NTSS does not capture information on changes to regimens during the course of treatment or the date when drug susceptibility testing results were received, and we were unable to assess this information directly.

In 2013, time from treatment start date to date of linked genotyping results was a median of 107 days (range 15–365 days). Thus, for 50% of cases, clinicians would have received genotyping results after the event time of this analysis (i.e., 90 days), which would diminish any potential influence on our findings. Moreover, removing pyrazinamide from regimens used to treat *M. bovis* TB cases would have not have affected time to culture conversion; rather, it would have prolonged it.

Concurrent and immunosuppressive conditions, such as diabetes mellitus, end-stage renal disease, and hematologic or reticuloendothelial malignancies, which might influence time to culture conversion, were not routinely collected during our study period. These variables were included as part of routine reporting in 2009 and might be helpful in future studies (*29*). Because the proportion of race/ethnicity differed by bacterial species, and some race/ethnicities have a higher prevalence of concurrent conditions (e.g., diabetes mellitus) that might influence time to culture conversion, we attempted to run a separate hazard model on the basis of race/ethnicity, but this variable did not satisfy the proportional hazards assumption.

When we restricted analysis to only Hispanic persons, *M. bovis* TB patients had similar hazards of converting sputum cultures to negative (HR 1.20, 95% CI 1.10–1.35) as in our adjusted model (Table 2). This finding suggests that self-identifying as Hispanic had no effect on our main finding that *M. bovis* TB cases had a higher hazards of converting sputum cultures to negative relative to *M. tuberculosis* TB cases. Although we attempted to control for bacillary burden by using a novel composite variable, smear grade

is not reported in NTSS, and our estimates on the effect of bacillary burden might be inaccurate.

Our findings should be interpreted with caution. Although we found earlier culture conversion for *M. bovis* TB patients than for *M. tuberculosis* TB patients, a larger proportion of *M. bovis* TB patients (8.9% vs. 5.1%) died during treatment. This finding suggests that earlier culture conversion does not necessarily lead to better clinical outcomes. Further laboratory studies should be conducted to better monitor and assess the time to sputum-culture conversion and clinical outcomes between these 2 *M. tuberculosis* complex species. If similar results are observed, further randomized clinical trials for treatment duration (and possibly treatment regimen) might be warranted.

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Use of Mass-Participation Outdoor Events to Assess Human Exposure to Tickborne Pathogens

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Mapping the public health threat of tickborne pathogens requires quantification of not only the density of infected host-seeking ticks but also the rate of human exposure to these ticks. To efficiently sample a high number of persons in a short time, we used a mass-participation outdoor event. In June 2014, we sampled ≈500 persons competing in a 2-day mountain marathon run across predominantly tick-infested habitat in Scotland. From the number of tick bites recorded and prevalence of tick infection with *Borrelia burgdoferi* sensu lato and *B. miyamotoi*, we quantified the frequency of competitor exposure to the pathogens. Mass-participation outdoor events have the potential to serve as excellent windows for epidemiologic study of tickborne pathogens; their concerted use should improve spatial and temporal mapping of human exposure to infected ticks.

The countryside (outdoors) represents a contemporary arena for recreation, and the benefits of such recreation to human health and well-being are widely recognized and strongly promoted by governments and other stakeholders. However, the countryside also harbors particular hazards that might be managed to minimize the threat they pose to countryside users. Among these hazards are numerous infectious diseases (e.g., Lyme borreliosis and leptospirosis) that are usually more abundant in the countryside than in urban areas. The public health burden of such infections can be defined as the product of the abundance of potential sources of infection (i.e., environmental hazards) and the frequency of human exposure (*1*). Although measuring both of these parameters presents difficulties, accurately quantifying the frequency of human exposure is particularly challenging.

Lyme borreliosis, caused by the spirochaete *Borrelia burgdorferi* sensu lato (s.l.), is a tickborne disease encountered primarily in the temperate regions of the Northern Hemisphere; in some countries, many thousands of cases are reported each year. For example, in the United States, ≈300,000 cases per year are estimated (*2*), and in the Netherlands, ≈25,000 new cases are reported each year (*3*). In the United Kingdom, Lyme borreliosis is less frequently reported; in 2014, the most recent annual data released by Public Health England (https://www.gov.uk/government/publications/lyme-borreliosis-epidemiology) and Health Protection Scotland (http://www.hps.scot.nhs.uk/giz/wrdetail.aspx?id = 65883&subjectid = 100&wrtype = 6) indicate almost 1,500 cases. In keeping with elsewhere in Europe, the incidence of cases in the United Kingdom has increased dramatically since the turn of the century and even now is considered a significant underestimation (*4*). The pathogenic potential of *Borrelia miyamotoi* was first reported in Russia in 2011; infections were most frequently manifested as an influenza-like illness (*5*). However, although the presence of this pathogen in *Ixodes* ticks across the Northern Hemisphere has been widely reported, reported cases of human disease are still rare (*6*) and have yet to be encountered in the United Kingdom. When estimating the public health burden of tickborne pathogens, the environmental hazard is represented by the abundance of infected ticks. Methods such as dragging and flagging for quantifying the abundance of questing *I. ricinus* nymphs and adults are well established and, although not without their shortcomings (*1*), have been widely adopted. However, methods quantifying human exposure to infected ticks are much more challenging. In the Netherlands, general practitioner consultations for tick bites have been used to monitor regional/national change in the frequency of tick bites (and thus tick activity) (*7*), but as most persons probably remove ticks themselves rather than rely on a general practitioner, this approach is a poor indicator of, at best, relative rather than absolute frequency.

Mass-participation events, varying from music festivals to ultramarathons, are now well-established features in the spectrum of recreation in the countryside. The structured nature of these events probably results in most participants interacting with the landscape they occupy in a predictable (in spatial and temporal terms at least) manner. Thus, from the perspective of infection epidemiology, mass-participation events provide opportunities to easily collect large numbers of samples (which would otherwise require considerable effort to accumulate) over a short period. In this study, we demonstrate the potential of this approach by using a mass-participation event to quantify the frequency with which those engaged in outdoor pursuits in tick-infested areas actually get bitten.

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Materials and Methods

Sample Collection

The Lowe Alpine Mountain Marathon (LAMM) is an annual long-distance mountain running event held in the Scottish Highlands. Teams of 2 runners each navigate mountainous terrain, carrying all their equipment for an overnight camp. To suit competitors with different levels of expertise and fitness, the event comprises 2 days of racing over several routes of varying lengths, typically 20–40 km. In June 2014, the LAMM took place around Wester Ross on the west coast of mainland Scotland (http://www.lamm.co.uk/2014/LAMM2014Map.jpg); the competition area covered ≈500 km^2. Racing on the first day was completed by 624 competitors, of whom 566 went on to complete racing on the second day. Before the event, the objectives and methods of our study were circulated to all competitors via a LAMM website (no longer available) and were reiterated to competitors in person the night before the race. As competitors passed through the finish area on the first day, they were given a 1.5-mL tube, containing 70% ethanol and labeled "1" and were asked to place any ticks they found on their bodies into the tube and then put the tube into a bin provided at the overnight midway camp. We also offered a tick-removal service, which proved popular and served to encourage wider participation. As competitors passed through the finish area on the second day, they were given an addressed envelope containing a 1.5-mL tube containing 70% ethanol and labeled "2." Competitors were asked to check themselves either immediately or when they returned home, to place any ticks found in the tube as before, and then to put the envelope containing the tube in the mail. After we processed the samples and collated the data, we posted a summary of survey results (at http://www.lamm.co.uk/2014/LAMM_LymeResults_2014.pdf), and the link was emailed to all competitors. This email also asked any competitor in whom Lyme borreliosis developed after the LAMM to contact the study team.

To assess if borreliae were an environmental hazard in the area and provide an indication of the scale of this hazard, we surveyed the competition area of the event for questing ticks by blanket dragging at 3 sites, specifically the day-1 assembly area (rough pasture used for sheep grazing) surveyed the night before the event, the overnight midway camp (heather, long grass), and pine woodland in Lochcarron, located ≈2 km from the assembly area (both surveyed during event) (Table). These efforts also served to increase awareness of the ongoing study throughout the event. Each site was surveyed for 30 minutes by repeatedly dragging a 1-m^2 wool blanket over the vegetation. After each drag, all ticks were removed from the blanket and placed in 70% ethanol.

Identification of Ticks and Determination of Infection Status

We recorded the life stages of all ticks and examined them microscopically to identify their species. A DNA extract was prepared from each tick and then incorporated as template into a real-time PCR, originally described for the detection of *B. burgdorferi* s.l. but subsequently found to have a specificity that includes *B. miyamotoi* (*8*). To delineate *Borrelia* species/genospecies, we incorporated as template extracts that yielded a product in this reaction into a *B. burgdorferi* s.l.–specific nested PCR (*9*) and a *B. miyamotoi*–specific real-time PCR (*10*). Products of the first of these reactions were sequenced, and unambiguous sequence data were used to determine *Borrelia* genospecies (*9*). Extraction/cross-contamination controls were co-processed with ticks at a ratio of 1:4. Each PCR incorporated positive and negative (reagents only) controls. PCRs were prepared in a dedicated laboratory in which PCR products were never handled.

Results

Survey

The species of all ticks encountered in our study was *I. ricinus*. On day 1 of the LAMM, 217 ticks were removed and submitted by 53 competitors, and on day 2 (or later by mail), 347 ticks were removed and submitted by 78 competitors. It is not known how many competitors submitted ticks on both days, but on the basis of a recorded 624 competitors on day 1 and 566 on day 2, we an estimate that a minimum of ≈8.5% of competitors on day 1 and 13.8% of competitors on day 2 were bitten by ticks. Significantly more competitors were bitten on day 2 than on day 1 (χ^2 8.47, 1 df, $p<0.01$). We collected 98 submissions containing 153 nymphs. A total of 44 (7.1%) competitors on day 1 and 54 (9.6%) competitors on day 2 were bitten by nymphs. We collected 75 submissions containing 411 larvae. A total of 25 (4.0%) competitors on day 1 and 50 (8.8%) competitors on day 2 were bitten by larvae. Both nymphs and larvae were removed from the skin of 16 (2.6%) competitors on

Table. Numbers of ticks collected and prevalence of *Borrelia burgdorferi* infections among those ticks at 3 Lowe Alpine Mountain Marathon sites, Scotland, 2014

Location	No. ticks collected	No. (%) nymphs *containing B. burgdorferi* DNA
Lochcarron	2 larvae,156 nymphs, 6 adults	11 (7.1)
Day-1 assembly area	0 larvae, 107 nymphs, 0 adults	16 (14.9)
Midway camp	0 larvae, 20 nymphs, 2 adults	2 (10.0)

day 1 and 26 (4.6%) competitors on day 2. Each submission contained 0–28 larvae and 0–9 nymphs, although most submissions contained either only 1 nymph or 1 larva.

Borrelia Infection Prevalence and Diversity among Ticks

Infection with *B. burgdorferi* s.l. was found in 3 (0.7%) of larvae and 19 (12.4%) of 153 nymphs that had fed on competitors. We were able to further characterize 16 of these ticks: 1 larva was infected with *B. afzelii*, 1 larva and 2 nymphs with *B. burgdorferi* sensu stricto, 7 nymphs with *B. valaisiana*, 2 nymphs with *B. garinii*, 1 nymph with *B. miyamotoi*; and 2 nymphs had mixed infections (1 with *B. valaisiana* and *B. garinii* and the other with *B. afzelii* and *B. garinii*). Infected larvae were removed from 2 competitors, and infected nymphs were removed from 16 competitors. In 4 instances, >1 tick removed from the same competitor was infected. A total of 10 infected ticks (3 larvae and 7 nymphs) were removed from 9 (1.4%) of the 624 day-1 competitors; whereas 12 infected nymphs were removed from 9 (1.6%) of the 566 day-2 competitors. No competitor reported development of Lyme borreliosis or symptoms compatible with *B. miyamotoi* infection subsequent to the LAMM.

Survey of the Environment

A total of 283 questing nymphal ticks were collected from the competition area and tested for the presence of DNA belonging to *Borrelia* spp. The overall prevalence of *B. burgdorferi* s.l. infection was 10.2% (29/283) (Table). We were able to further characterize infections in 11 of these ticks, finding 7 to be infected with *B. afzelii*, 3 with *B. garinii*, and 1 with *B. valaisiana*. The prevalence of infection among nymphs removed from humans did not differ significantly from that among questing ticks (χ^2 0.75, 1 df, p = 0.39).

Discussion

The presence of ticks at the site of the 2014 LAMM was entirely expected, as was presence of *B. burgdorferi* s.l. infections in these ticks. In a recent survey of 25 sites across Scotland, primarily in the Highlands, infected ticks were found at all sites (*11*). The overall prevalence of *B. burgdorferi* s.l. infections in questing nymphs and in nymphs removed from competitors (48/436, 11.0%) and the diversity of *B. burgdorferi* s.l. genospecies encountered was akin to that previously reported in Scotland and elsewhere in the United Kingdom (*8*,*11*). The presence of *B. miyamotoi* in Scotland has not been reported previously, but a study of questing nymphs in England suggested that the pathogen is widespread, albeit at a very low prevalence (3/954, 0.3%) (*12*).

Perhaps the most unexpected observation was the high proportion of competitors bitten by ticks; our estimates are that 8.5% were bitten on day 1 and 13.8% on day 2. Of 131 competitors who reported being bitten by ticks, 33 (≈25%) found larvae only. This life stage is often overlooked with regard to *B. burgdorferi* s.l. transmission, but our detection of *B. burgdorferi* s.l. DNA in 0.7% of larvae from competitors supports the work of others (*13*–*15*), indicating that although rates of infection may be relatively low among larvae, ticks at this life stage should not be disregarded as a source of Lyme borreliosis. Low prevalence of *B. miyamotoi* infections in *I. ricinus* larvae collected in mainland Europe has been demonstrated (*15*). Our failure to detect this species in any of the 411 larvae we tested indicates that, on the event site at least, infection of larvae is extremely rare. The remaining 98 submissions, representing 75% of all those bitten by ticks, found either nymphs only or both nymphs and larvae. Nymphs have far greater potential to transmit *B. burgdorferi* s.l.; in this study, prevalence of infection among nymphs was ≈18 times higher than that among larvae. Of 98 nymph submissions, 16 (16%) were infected with either *B. burgdorferi* s.l. or *B. miyamotoi*; 1.1% of day-1 competitors and 1.6% of day-2 competitors removed infected ticks from their bodies.

Over the 2 days of the LAMM, competitors assembled at the camping area at 6 PM on the evening before the start and departed after finishing the race 2 days later. Given that we encountered ticks not just on the LAMM course but also in the camping area used by the competitors when not racing, we can reasonably assume that the only periods when competitors were not exposed to ticks was when they were inside their tents. We therefore estimate that through the 48 hours of the event, 624 competitors were exposed to ticks for ≈32 hours, a total of ≈20,000 competitor-hours. The total of 564 tick bites occurring during this period indicate 1 bite every 35 competitor-hours. Of these, 153 were from nymphs; thus, 1 nymph tick bite occurred every 131 competitor-hours; a bite from an infected nymph occurred once every 1,051 competitor-hours.

These data provide an early quantification of the frequency of human exposure to *B. burgdorferi* s.l. in the vicinity of the LAMM and, given the estimated abundance of nymphal ticks in this area and the prevalence of *B. burgdorferi* s.l. infections among these ticks, may represent values encountered more widely across Scotland and other parts of the United Kingdom (*8*,*11*). Extrapolation of these data may offer a tentative insight into frequency of human exposure in similar tick-infested habitat across the country. However, as well as caveats associated with geographic/spatial variation in the environmental hazard posed by *B. burgdorferi* s.l., other determinants of tick exposure need also to be considered, including anthropogenic determinants. For example, the degree to which competitors' behavior was representative of countryside users at large (or a specific cohort thereof) is unknown; the infrequency with which LAMM competitors used clear footpaths may have resulted in their encountering questing ticks more often,

and their light apparel (many competitors wore shorts and short-sleeved tops) may have enabled attachment of more ticks. In addition, abiotic factors such as the influence of local weather conditions on tick questing behavior during the LAMM and the topography of the course, sections of which were at relatively high altitudes where ticks are less abundant (*16*), need to be considered. Of note, we observed more frequent tick bites on the second day of competition, despite persistent light rain throughout the morning, an observation in keeping with previous reports that ticks remain active in rain and that rainfall is associated with increased tick activity (*17*). However, we cannot rule out that this observation resulted from competitors simply being more vigilant after the competition finished.

Various approaches to exploring the frequency of human exposure to ticks have previously been taken. These approaches vary from the quantification of ticks accumulated on fabricated leggings worn by investigators moving over/through tick-infested vegetation (thus, rate of encounter rather than attachment) (*1*,*18*) to assessment of the number of patients seeking help from medical staff for tick removal; in 1 such survey of visitors to a popular woodland site in southern England during April–October 1996 and 1997, ticks were removed from almost 1,100 persons (*19*). However, only very few studies have attempted to quantify the rate at which users of tick-infested habitat actually get bitten. One survey, of 568 soldiers at an outdoor training base in Germany, recorded 710 tick bites during April–September 2009, a mean incidence rate of 2.3 bites/1,000 person-days (*20*). More recently, a survey of 931 scouts attending summer camps in Belgium yielded a mean incidence rate of 22.8 tick bites/1,000 person-days (*21*). Both of these incidence rates are markedly lower than that reported in the current study, which equates to 677 bites/1,000 competitor-days. Why such variation exists is unclear; it may be artifactual, resulting from differences in the efficiency of tick detection in 3 studies, or it may be genuine. If the latter, it is worth considering that our survey was conducted in early June, when the abundance of immature ticks is probably near its seasonal peak (*22*), whereas the surveys in Germany and Belgium extended over several months, during some of which tick abundance is probably reduced. Indeed, in the study in Germany, >95% of all tick bites occurred during 3 of the 6 months (April–September) surveyed (*20*). Also, in Belgium, marked differences in incidence rates were observed between camps; a range of 0–97.9 bites/1,000 person-days were recorded (*21*). None of the 3 surveys under discussion included accurate estimates of questing tick densities at the survey sites; however, given that the questing tick densities at the 3 LAMM sites surveyed were not unusually high (mean of 189 ticks/person-hour, which equates to 98/100 m^2 (*23*), it is unlikely that the ≈100-fold variation in mean incidence rate of tick bites between our study and that of Faulde et al. (*20*) is solely a reflection of variation in local tick abundance. Perhaps more pertinent is that the participants of the study in Germany wore battle dress uniforms and were provided with arthropod-repellent skin cream, whereas the LAMM competitors wore light apparel, frequently shorts and short-sleeved tops, thus making it easier for ticks to access their skin (and possibly attracting ticks more by unimpeded release of attractant molecules from the skin). De Keukeliere et al. (*21*) did not provide details of what the participants in their study wore or the nature of their activities, but given that they were all scouts 8–16 years of age, it is reasonable to assume their clothing was more akin to that of LAMM competitors than that of German soldiers.

That none of the competitors reported clinical symptoms compatible with either *B. burgdorferi* s.l. or *B. miyamotoi* infections subsequent to the LAMM is most likely the result of the well-established prophylactic benefits of removing ticks within a few hours of attachment. However, it may also reflect a low transmission rate of *B. burgdorferi* s.l. from ticks to humans (*19*,*24*) or a low incidence of clinical manifestations among those infected (*25*,*26*).

In summary, our study demonstrates how mass-participation outdoor recreational events can be used to assess human exposure to dangers such as tickborne pathogens. By using one such event, we were able to survey a large number of persons very efficiently; we obtained more samples in just 2 days than previous studies with similar aims (*19*,*20*) obtained in 6 months. Mass-participation outdoor recreational events are now more diverse and popular than ever; for example, thousands of orienteers and mountain/hill/trail runners participate in events every weekend, and vast numbers attend music or arts festivals throughout the summer. Furthermore, participants generally have a strong connection with the outdoors and so are probably willing contributors to scientific studies linked to nature. A refinement to our approach would be to track more precisely the movement of persons through the landscape. This tracking could be achieved by focusing on events at which participants follow a fixed course or wear GPS (global positioning system) tracking devices (as is becoming increasingly common), akin to a study quantifying the risk for tick infestation of dogs (*27*).

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Miss Hall is pursuing a PhD degree at the University of Salford. Her research is focused on better understanding the drivers underlying the public health threat of tickborne pathogens in the United Kingdom, in particular *B. burgdorferi*.

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Pulmonary Nontuberculous Mycobacteria–Associated Deaths, Ontario, Canada, 2001–2013

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Survival implications of nontuberculous mycobacterial pulmonary disease (NTM-PD) and NTM pulmonary isolation without disease (NTM-PI) are unclear. To study deaths associated with NTM-PD and NTM-PI and differences in survival between them, we conducted a population-based cohort study of persons with microbiologically defined NTM-PD or NTM-PI diagnosed during 2001–2013 in Ontario, Canada. We used propensity score matching and Cox proportional hazards models to compare survival. Among 9,681 NTM-PD patients and 10,936 NTM-PI patients, 87% and 91%, respectively, were successfully matched with unexposed controls. Both NTM-PD and NTM-PI were associated with higher rates of death for all species combined and for most individual species. Compared with NTM-PI, NTM-PD was associated with higher death rates for all species combined, *Mycobacterium avium* complex, and *M. xenopi*. NTM-PD and NTM-PI were significantly associated with death, NTM-PD more so than NTM-PI.

Nontuberculous mycobacterial pulmonary disease (NTM-PD) is an increasingly common problem (*1*–*3*) that is associated with substantially impaired quality of life (*4*) and is difficult and costly to treat (*5*,*6*). At the population level, patients with NTM-PD have been poorly characterized in general, and their survival is not well studied. Studies from individual clinical programs have identified prognostic factors (*7*–*10*), but estimates of survival are undoubtedly affected by referral bias and therefore cannot be generalized to all NTM-PD patients. One population-based study of survival in NTM-PD patients has been reported, but it did not include controls without NTM, so NTM-attributable death could not be determined (*11*). Furthermore, differences in phenotypes between NTM-PD patients in Europe and North America (*12*) suggest that death rates might differ substantially between these regions. In the United States, a study of Medicare beneficiaries estimated a 40% higher risk for death in persons with NTM-PD than in persons without it, but the authors used a nonvalidated case definition and excluded patients <65 years of age and patients enrolled in health maintenance organizations (*3*).

In studying all residents of Ontario, Canada, who had NTM-PD, we sought to describe their clinical characteristics, estimate their survival, and determine whether patients with NTM-PD have higher rates of death than population age-, sex-, and propensity-matched unexposed controls. In addition, we sought to compare survival of patients with NTM pulmonary isolation but not disease (NTM-PI) with patients who had NTM-PD, as well as survival for patients with different NTM species.

Methods

We conducted a population-based matched cohort study using linked health administrative data and mycobacteriology as described previously (*13*), including all Ontario residents with incident pulmonary NTM isolation diagnosed during 2001–2013, and unexposed controls matched by age, sex, index date, and propensity score (*14*). The responsible institutional review committees approved this study. Additional details about the data sources and methods are provided in the online Technical Appendix (http://wwwnc.cdc.gov/EID/article/23/3/16-1927-Techapp1.pdf).

Using microbiological criteria from current guidelines (*5*), we defined 2 mutually exclusive groups. One positive sputum sample defined NTM-PI, whereas >1 positive sputum sample for the same species or 1 positive bronchoscopic or biopsy specimen defined NTM-PD. We disregarded *Mycobacterium gordonae* isolates and excluded

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persons with prior (1998–2000) NTM isolation. We calculated 12 propensity scores (estimating the patient-level likelihood of species–condition combinations), 1 for each species–condition combination of interest, comprising the species groups *M. avium* complex (MAC), *M. xenopi*, *M. fortuitum*, M. *abscessus*, *M. kansasii*, and other, according to the condition-states NTM-PI and NTM-PD. We sought to match each patient with NTM (exposed person) to an Ontario resident without NTM (unexposed control) who shared the age (years), sex, and index date (± 90 days), and had a propensity score value within 0.2 × SD of the exposed patient (*15*). Index date was date of first positive culture for exposed patients and was randomly assigned to potential unexposed controls by using a random number generator.

We characterized our cohort by demographics, underlying conditions, and healthcare utilization. Primary analyses compared survival of propensity score–matched exposed with unexposed persons, for each species–condition group (e.g., patients with MAC NTM-PD vs. their population-matched unexposed controls). We also compared, for individual NTM species-groups and all species-groups combined, survival of patients with any isolation (NTM-PI or NTM-PD) to their propensity score–matched controls and survival of all patients with NTM-PD or NTM-PI (without matching). We also calculated standardized mortality ratios, using age- (in 5-year strata) and sex-specific all-cause death rates in the Ontario general population during the study period (2001–2013), as an intuitive mortality risk assessment without full adjustment. To study whether NTM-PD with >1 NTM species (multispecies NTM-PD) affected survival, we compared the survival of NTM-PD patients infected with 1 species with survival of patients who fulfilled criteria for >1 species. Finally, we examined associations between demographic and clinical factors with death.

Follow-up began on the index date and ended at death or end of the study period, whichever came first. Survival analyses comprised Cox proportional hazards models. Including low-frequency covariates (Table 1) in propensity score calculation led to substantially fewer NTM patients successfully matching to an unexposed person. These covariates were explored for inclusion by using a Hosmer-Lemeshow approach (inclusion if ≥10% effect on point estimate [*18*]); none were retained. Comparisons between groups that were not propensity-matched (NTM-PD vs. NTM-PI and single-species vs. multispecies NTM-PD) included all Ontario residents with NTM species–conditions of interest and were adjusted for age, sex, and covariates used to characterize the cohort in our study. For survival analyses of single-species versus multispecies NTM-PD, we addressed immortal time

Table 1. Characteristics of patients with NTM pulmonary disease and matched persons without NTM for MAC, *Mycobacterium xenopi*, and *M. abscessus*, Ontario, Canada, 2001–2013*

	MAC			*M. xenopi*			*M. abscessus*		
Characteristic	Disease, n = 5,543	Control, n = 5,543	SDM	Disease, n = 1,975	Control, n = 1,975	SDM	Disease, n = 201	Control, n = 201	SDM
Female sex, %	53	53	0	45	45	0	58	58	0
Median age, y (IQR)	70 (58–78)	70 (58–78)	0	70 (58–78)	70 (58–78)	0	64 (47–74)	64 (47–74)	0
Underlying condition, %									
Asthma	31	29	0.06	36	31	0.1	25	31	0.13
COPD	45	52	0.14	51	56	0.11	32	34	0.04
Diabetes	19	25	0.15	21	24	0.09	12	15	0.1
Rheumatoid arthritis	3	3	0.01	3	3	0.01	<3†	<3†	0.13
Chronic kidney disease	7	9	0.05	8	8	0	5	5	0
GERD	16	19	0.05	17	18	0.02	11	13	0.05
Bronchiectasis	10	5	0.17	7	5	0.06	9	8	0.04
Interstitial lung disease	5	4	0.06	6	4	0.11	6	4	0.1
Lung cancer	5	3	0.09	7	4	0.13	4	5	0.07
HIV infection‡	2	0.2	0.18	2	0	0.18	0	0	
Solid organ transplant‡	0.5	0.1	0.07	1	<0.3†	0.13	3	0	0.25
BMT‡	0.4	<0.1	0.06	1	<0.3†	0.12	<3†	0	0.14
Cystic fibrosis‡	0.5	<0.1	0.07	0.4	<0.3†	0.04	8	0	0.42
Prior tuberculosis‡	2	0	0.18	2	<0.3†	0.19	4	0	0.29
Hospitalizations§	0.31 ± 0.76	0.32 ± 0.77	0.01	0.39 ± 0.84	0.37 ± 0.84	0.02	0.29 ± 0.68	0.30 ± 0.71	0.02
ED visits§	0.85 ± 1.15	0.80 ± 1.56	0.03	0.88 ± 1.20	0.80 ± 1.51	0.06	0.55 ± 0.88	0.69 ± 1.37	0.12
ACG diagnoses	9.7 ± 3.8	9.5 ± 3.8	0.05	10.1 ± 4.0	9.8 ± 3.9	0.06	8.6 ± 3.9	8.5 ± 4.2	0.03

*Matched according to age (years), sex, index date (± 90 d), and propensity score (estimating the patient-level likelihood of species-specific NTM pulmonary disease) value within 0.2 × SD of the exposed patient. NTM pulmonary disease was defined as the presence of >1 positive sputum sample for the same species or 1 positive bronchoscopic or biopsy specimen. Controls were persons without NTM matched by age, sex, index date, and propensity score. ACG; adjusted clinical group diagnoses using the ACG case mix system (*16*); BMT, hematopoietic stem cell transplant; COPD, chronic obstructive pulmonary disease; ED; emergency department; GERD, gastresophageal reflux disease; IQR, interquartile range; MAC, *Mycobacterium avium* complex; NTM, nontuberculous mycobacteria; SDM; standardized difference of the mean (value of <0.1 generally considered not significant) (*17*). Empty cells indicate value undefined.

†Range reported because of small cell size (direct or by inference), which in accordance with privacy regulations cannot be reported.

‡Baseline characteristics not included in the propensity score because of their effect to substantially reduce successful matching of exposed cases with unexposed controls. Inclusion of these variables as covariates was explored, but none significantly altered the hazard ratio point estimates.

§Number of events in year before index date.

bias (time to second species infection inflating survival of patients with multispecies NTM-PD) using status of single-species versus multispecies NTM as a time-varying covariate. Secondary survival analyses excluded patients who died within 30 days after NTM index date, assuming that early death was unrelated to NTM.

Results

During the 13-year study period, 20,617 Ontario residents had incident NTM isolation from respiratory tract specimens: 10,936 (53%) with NTM-PI and 9,681 (47%) with NTM-PD. Propensity score matching was successful for 9,967 (91%) NTM-PI patients and 8,469 (87%) NTM-PD patients. Compared with matched patients, patients who could not be matched to unexposed controls were older (NTM-PI, median 74 vs. 64 years, p<0.001; NTM-PD, median 72 vs. 70 years, p<0.001) and had higher frequencies of underlying conditions with higher mean adjusted clinical group case mix (*16*) numbers (NTM-PI 12.6 vs. 8.9, p<0.001; NTM-PD 12.4 vs. 9.8, p<0.001) (online Technical Appendix Table 1). In addition, unmatched patients had substantially lower survival than did matched patients at 1 year (76.1% vs. 91.0%) and 5 years (46.3% vs. 76.0%) for all NTM-PI and at 1 year (75.3% vs. 85.7%) and 5 years (47.7% vs. 65.4%) for all NTM-PD.

We observed small differences in sex distribution by species, whereby NTM-PD with MAC and *M. abscessus* was seen more commonly in female patients and the other NTM species were seen more commonly in male patients (Table 1; Table 2, https://wwwnc.cdc.gov/EID/article/23/3/16-1927-T2.htm; online Technical Appendix Tables 2–4). Combining all species, the sexes were similarly represented for NTM-PD and NTM-PI. The median (interquartile range) age ranged from 60 (43–74) to 70 (58–78) years for the different species–condition groups and for combining species was 65 (49–77) years for NTM-PI and 70 (58–78) years for NTM-PD. Patients had a high prevalence of underlying conditions by adjusted clinical group numbers (7.3–10.1), including asthma (25%–36%), chronic obstructive pulmonary disease (COPD; 25%–52%), diabetes (12%–24%), chronic kidney disease (3%–8%), and gastresophageal reflux disease (11%–20%), ranging by species–condition groups. Covariates were generally balanced between the matched groups.

Kaplan-Meier plots for any NTM (NTM-PD or NTM-PI), including both matched and unmatched patients, by species group, revealed apparently distinct death rates; survival was highest for patients with *M. abscessus* and lowest for patients with *M. kansasii* (Figure). In this crude survival comparison, uncontrolled for age, sex, or any other variables,

Table 2. Characteristics of patients with NTM pulmonary isolation and matched persons without NTM for MAC, *Mycobacterium xenopi*, and *M. abscessus*, Ontario, Canada, 2001–2013*

	MAC			*M. xenopi*			*M. abscessus*		
Characteristic	Isolation, n = 5,242	Control, n = 5,242	SDM	Isolation, n = 2,693	Control, n = 2,693	SDM	Isolation, n = 162	Control, n = 162	SDM
Female sex, %	51	51	0	48	47	0	49	49	0
Median age, y (IQR)	65 (49–76)	65 (49–76)	0	65 (48–76)	65 (48–76)	0	60 (43–74)	60 (43–74)	0
Underlying condition, %									
Asthma	29	25	0.09	31	26	0.1	25	20	0.12
COPD	36	41	0.1	38	42	0.09	25	26	0.01
Diabetes	18	23	0.12	19	21	0.06	15	16	0.03
Rheumatoid arthritis	3	2	0.06	3	3	0.01	<4†	5	0.09
Chronic kidney disease	6	7	0.05	7	8	0.03	5	6	0.05
GERD	14	17	0.08	15	17	0.05	14	13	0.02
Bronchiectasis	7	4	0.13	6	4	0.09	6	5	0.03
Interstitial lung disease	3	2	0.06	3	3	0.03	<4†	4	0.11
Lung cancer	2	2	0.02	2	2	0.04	<4†	<4†	0.18
HIV infection‡	1	0.2	0.13	2	<0.2†	0.19	0	0	
Solid organ transplant‡	0.3	0.3	0	0.5	<0.2†	0.07	0	<4†	0.11
BMT‡	0.1	0.1	0	<0.2†	<0.2%†	0.04	0	0	
Cystic fibrosis‡	0.4	0.1	0.05	0.2	0	0.07	5	<4†	0.27
Prior tuberculosis‡	3	<0.1†	0.23	3	<0.2%†	0.25	<4†	0	0.19
Hospitalizations§	0.31 ± 0.77	0.30 ± 0.76	0.01	0.33 ± 0.78	0.31 ± 0.78	0.02	0.19 ± 0.53	0.23 ± 0.60	0.09
ED visits§	0.83 ± 1.22	0.79 ± 1.79	0.02	0.81 ± 1.21	0.77 ± 1.59	0.03	0.49 ± 0.88	0.46 ± 1.08	0.03
ACG diagnoses	9.0 ± 4.1	8.7 ± 4.1	0.06	9.1 ± 4.2	8.8 ± 4.2	0.08	7.3 ± 4.3	7.4 ± 4.4	0.01

*Matched according to age (years), sex, index date (± 90 d), and propensity score (estimating the patient-level likelihood of species-specific NTM pulmonary isolation) value within 0.2 × SD of the exposed patient. NTM pulmonary isolation was defined as the presence of 1 positive sputum specimen. Controls were persons without NTM matched by age, sex, index date, and propensity score. ACG, adjusted clinical group diagnoses using the ACG case mix system (*16*); BMT, hematopoietic stem cell transplant; COPD, chronic obstructive pulmonary disease; ED, emergency department; GERD, gastresophageal reflux disease; IQR, interquartile range; MAC, *Mycobacterium avium* complex; NTM, nontuberculous mycobacteria; SDM, standardized difference of the mean (value <0.1 generally considered not significant) (*17*). Blank cells indicate value undefined.
†Range reported because of small cell size (direct or by inference), which according to privacy regulations cannot be reported.
‡Baseline characteristics not included in the propensity score because of their effect to substantially reduce successful matching of exposed cases with unexposed controls. Inclusion of these variables as covariates was explored, but none significantly altered the hazard ratio point estimates.
§Number of events in year before index date.

we found a statistically significant difference among curves (p<0.001, log-rank) and significant differences in most pairwise comparisons between species.

Compared with age-, sex-, and propensity-matched unexposed controls, 1- and 5-year survivals were numerically lower for patients with NTM in all groups, regardless of species or condition (NTM-PI or NTM-PD) (Table 3). Hazard ratios (HRs) for death were elevated for all species–condition groups, but those for *M. abscessus* isolation (HR 1.39, 95% CI 0.94–2.07), and *M. fortuitum* disease (HR 1.25, 95% CI 0.96–1.63) were not statistically significant.

As illustrated by the standardized mortality ratios, adjusted for sex and 5-year age stratum, death rates were increased above expected for the Ontario general population for all groups compared (Table 4). In adjusted comparisons, patients with NTM-PD (vs. NTM-PI) had higher death rates for all species combined, as well as for MAC and *M. xenopi* individually (Table 4). For the other species groups, although the HRs were similar in magnitude to that for MAC, death rates were not significantly higher. Compared with the 9,061 patients who had single-species NTM-PD, the 620 patients with multispecies NTM-PD (any combination of NTM species) had higher rates of death (HR 1.19, 95% CI 1.04–1.34). Death rates also were higher for the subgroup of MAC plus *M. xenopi* (n = 354) versus either species alone (n = 8,059) (HR 1.23, 95% CI 1.04–1.45). The sample size was inadequate to assess other species combinations. In the multivariable analysis of baseline factors for association with death among all patients with incident NTM-PD (matched and unmatched patients), increasing age, male sex, low income, and underlying conditions were all associated with reduced survival (Table 5). Compared with MAC (reference species group), *M. xenopi* was the only species associated with a significantly higher death rate. The results of secondary survival analyses that excluded patients who died within 30 days did not differ significantly from any primary survival analyses.

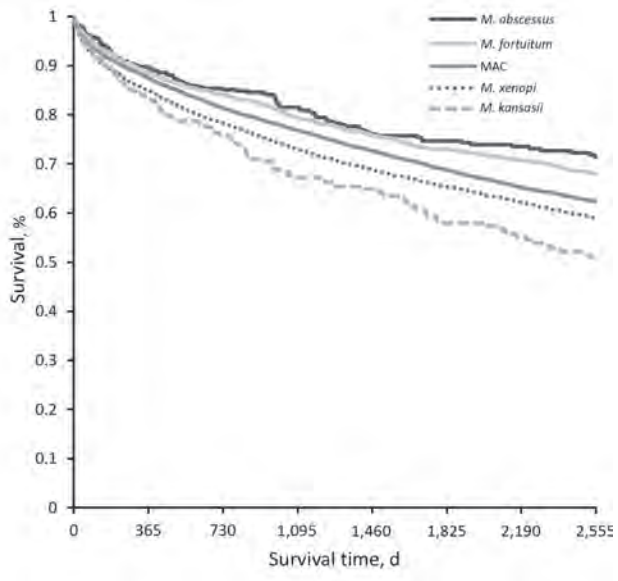

Figure. Kaplan-Meier survival curves for any pulmonary NTM isolation, by species group, Ontario, Canada, 2001–2013. Curve comprises all matched and unmatched patients identified during the study period. There is a statistically significant difference among curves (p<0.001, log-rank) in crude survival comparison, uncontrolled for any other variables. Differences between individual species pairs statistically significant (p<0.00005) for all pairs except *Mycobacterium abscessus* versus *M. fortuitum* (p = 0.19), *M. abscessus* versus *Mycobacterium avium* complex (p = 0.14), and *M. fortuitum* versus *Mycobacterium avium* complex (MAC) (p = 0.50).

Discussion

Our population-based cohort study matched >18,000 patients who had microbiologically confirmed pulmonary NTM to unexposed controls and clearly demonstrates an increased risk for death with both NTM-PD and NTM-PI. The veracity of this prognostic information is supported by the rigorous survival comparison with unexposed persons matched by age, sex, and the propensity to have NTM. The relatively small sample sizes might account for the fact that we did not observe a significant risk for death in some individual subgroups (NTM-PI with *M. abscessus* and NTM-PD with *M. fortuitum*) compared with unexposed controls.

Previous studies demonstrated high rates of death with NTM (*7*–*11*), but none compared with matched population-based controls. In addition, most prior studies comprised patients from NTM clinics, which permitted careful clinical characterization but most likely led to substantial bias in patient selection (*7*,*8*,*10*). Although our 5-year mortality estimates (26.6% for NTM-PI and 36.9% for NTM-PD) were of generally similar magnitude to prior studies (*7*–*11*), parsing those studies by cohort type reveals that those comprising single NTM clinics tended to report lower rates of death (*7*,*8*,*10*). In the single-clinic study from the United States, which had the lowest 5-year death rate of 18% (*10*), the median age at diagnosis was 55 years, compared with 70 years in our study, which probably explains much of the difference in death. The 2 single-clinic studies from Japan, comprising exclusively patients with MAC pulmonary disease (MAC-PD), reported 5-year death rates of 23.1% and 23.9% (*7*), compared with 33.5% for MAC-PD patients in our study, despite similar ages among the 3 studies. In 1 of the studies, treated MAC-PD patients had a 5-year risk for death of 22.2%, whereas untreated chronic MAC-PD patients had a 5-year risk for 33.3% (*8*), similar to the patients in our study. Perhaps most MAC-PD patients in Ontario have untreated chronic disease, whereas patients in NTM clinics are more likely to be treated, which might reduce their risk for death.

A comprehensive population-based study from Denmark observed death rates of 39.7% for MAC-PD and

Table 3. Survival estimates for patients with pulmonary NTM and for matched controls, Ontario, Canada, 2001–2013*

	Any NTM (isolation or disease)			NTM isolation only			NTM disease		
Group	Total	5-y survival, %	HR (95% CI)	Total	5-y survival, %	HR (95% CI)	Total	5-y survival, %	HR (95% CI)
NTM	18,436	71.3	1.47 (1.42–1.51)	9,967	76.1	1.33 (1.27–1.39)	8,469	65.6	1.63 (1.56–1.70)
Control	18,436	80.8	1.00 (ref)	9,967	82.6	1.00 (ref)	8,469	78.7	1.00 (ref)
MAC	10,785	71.2	1.45 (1.39–1.51)	5,242	75.9	1.33 (1.25–1.41)	5,543	66.7	1.57 (1.48–1.66)
Control	10,785	80.4	1.00 (ref)	5,242	82.3	1.00 (ref)	5,543	78.5	1.00 (ref)
M. xenopi	4,668	68.3	1.54 (1.45–1.64)	2,693	74.5	1.32 (1.22–1.44)	1,975	59.9%	1.84 (1.69–2.01)
Control	4,668	79.9	1.00 (ref)	2,693	81.5	1.00 (ref)	1,975	77.7%	1.00 (ref)
M. fortuitum	890	76.4%	1.39 (1.21–1.60)	654	78.8	1.47 (1.24–1.73)	236	69.7	1.25 (0.96–1.63)
Control	890	84.9%	1.00 (ref)	654	86.4	1.00 (ref)	236	80.7	1.00 (ref)
M. abscessus	363	80.5	1.45 (1.09–1.92)	162	82.2	1.39 (0.94–2.07)	201	79.2	1.49 (1.00–2.21)
Control	363	86.6	1.00 (ref)	162	85.7	1.00 (ref)	201	87.3	1.00 (ref)
M. kansasii	236	60.2	2.29 (1.76–2.97)	92	55.3%	2.02 (1.37–3.00)	144	63.5	2.53 (1.78–3.58)
Control	236	84.0	1.00 (ref)	92	85.3%	1.00 (ref)	144	83.0	1.00 (ref)
Other species	1,494	77.7	1.28 (1.13–1.43)	1124	80.0%	1.20 (1.04–1.38)	370	70.7	1.51 (1.22–1.88)
Control	1,494	82.4	1.00 (ref)	1124	83.2%	1.00 (ref)	370	79.8	1.00 (ref)

*Matched by age (years), sex, index date (± 90 d), and propensity score (estimating the patient-level likelihood of species–condition combinations) value within 0.2 × SD of the exposed patient. NTM isolation was defined as 1 positive sputum specimen for NTM. NTM disease was defined as >1 positive sputum for the same species or 1 positive bronchoscopic or biopsy specimen. Controls were persons without NTM matched by age, sex, index date, and propensity score. HR, hazard ratio; MAC, *Mycobacterium avium* complex; NTM, nontuberculous mycobacterium; ref, referent.

51.0% for *M. xenopi* disease, compared with 36.3% and 43.2% in our study. The higher rates of death in the Denmark study are interesting considering that the cohort was younger and had a smaller proportion of patients with COPD, diabetes, and renal disease. However, because the Denmark study did not provide detailed clinical characteristics and comprised approximately one third *M. gordonae* patients (*11*), comparing that cohort with the cohort in our study is difficult. Despite methodologic differences, the odds ratio (OR) for death in a Medicare-based study in the United States (OR 1.4, 95% CI 1.3–1.6) (*3*) was similar to our HR for death in survival analysis (HR 1.63, 95% CI 1.56–1.70). The US study comprised only patients who were ≥65 years, not enrolled in a health maintenance organization, and identified with NTM infection using codes from the International Classification of Diseases, Ninth Revision (ICD-9). Restricting our cohort to patients ≥65 years of age would exclude >37% of all patients with NTM-PD. Although the use of ICD-9 codes to identify patients with NTM infection has not been validated, this method appears to be specific but relatively insensitive (*20*), introducing bias depending on characteristics of NTM-infected patients not detected by this method.

The risk for death we observed with NTM-PD was generally greater than with NTM-PI, which was significant for all species combined, MAC, and *M. xenopi*. The lack of a significant difference in survival between NTM-PD and NTM-PI for the other species might have reflected the relatively small number of cases. Our results are consistent with findings in the population-based study from Denmark (*11*). In the Denmark study, 709 patients with 1 positive specimen tended to survive longer than the 238 patients with 2 or 3 positive specimens, who in turn tended to survive longer than the 335 patients who had >3 positive specimens ($p = 0.07$). The difference in statistical significance probably is due to sample size because our study had far more patients.

The significantly higher death rate for patients who had even 1 positive sputum specimen for MAC or *M. xenopi* is of particular interest. In some cases a positive sputum sample could be insignificant, representing contamination or transient presence of the organism, which would presumably not be associated with increased risk for death. In other cases, a positive sputum sample might represent the 1-time identification of a chronically present organism, which might or might not be causing significant disease. In this latter group, the presence of only 1 positive sample could be explained either by inadequate sampling or difficulty in identifying a true pathogen present in low numbers. Either way, some patients designated as having NTM-PI probably had true disease. The lack of data about negative cultures precludes further exploration of this issue.

As with others' findings, we identified variable survival among patients with different NTM species. Our unadjusted analyses are consistent with the Denmark study; patients with *M. abscessus* and *M. fortuitum* had lower death rates than patients with MAC, who in turn had lower death rates than patients with *M. xenopi* (*11*).

However, in our adjusted analysis, survival with *M. abscessus* and *M. fortuitum* pulmonary disease did not differ from survival with MAC disease. The worse crude survival observed with *M. xenopi* and *M. kansasii* persisted in adjusted analyses only for *M. xenopi*. The reason for the worse prognosis with *M. xenopi*, despite adjustment for age, sex, and underlying conditions, is not clear. There could be residual confounding related to cavitation and COPD, both commonly present with *M. xenopi* disease. Patients with *M. xenopi* disease have far higher rates of cavitation than do patients with MAC (46% vs. 16%; p = 0.01) (*21*), and cavitation is consistently associated with death (*7*,*8*,*10*). *M. xenopi* is also associated with COPD (*11*), and although both studies adjusted for COPD, the severity of COPD could be greater among patients with *M. xenopi* infection. Inadequately measured covariates could potentially explain the poor survival among patients infected with *M. xenopi*. Alternatively, perhaps *M. xenopi* disease is a more lethal condition. The lower survival observed with *M. kansasii* (compared with MAC) in unadjusted analysis, which did not persist in adjusted analysis, is most likely explained largely by the high proportion of male patients, COPD, and HIV infection among *M. kansasii* patients. Regardless, the adjusted analysis did not find that *M. kansasii* was associated with higher death rates than MAC, perhaps as expected because the former is believed to be the most readily curable of the NTM pulmonary pathogens (*5*), despite a high prevalence of cavitary disease (*22*).

The analysis of risk factors for death among patients with NTM-PD provided results generally consistent with those of previous studies. As in our study, increasing age (*7*,*11*), male sex (*3*,*7*,*11*), and underlying conditions (*3*,*11*) are repeatedly identified as risk factors for death. Other factors, including COPD, asthma, bronchiectasis, and other diseases, have been less studied. Our finding that COPD is associated with death (HR 1.38, 95% CI 1.29–1.48) seems plausible, not only because of the death intrinsic to COPD, but also because fibrocavitary NTM-PD develops more often in patients with COPD (*5*), and cavitation is associated with lower survival (*7*,*8*,*10*). Our finding that asthma is associated with a lower risk for death (HR 0.89, 95% CI 0.83–0.95) is perhaps surprising; it might be confounded by an association between asthma and nodular bronchiectatic NTM-PD, which has a better prognosis than cavitary disease (*7*,*8*,*10*). Although 1 previous study reported that NTM-PD patients with asthma had a higher risk for death (OR 1.7, 95% CI 1.1–2.7) (*3*), NTM-PD was identified by ICD-9 coding, which might overlook a large proportion of NTM-PD and thus introduce bias. The presence of bronchiectasis in NTM-PD presumably makes cavitation less likely to be present, and so our finding of lower death rates in the presence of bronchiectasis (HR 0.77, 95% CI 0.70–0.84) seems plausible and is consistent with a prior report

Table 4. Survival estimates for patients with incident pulmonary NTM disease and with NTM isolation, Ontario, Canada, 2001–2013*

Species, group	Total	1-y survival, %	5-y survival, %	SMR† (95% CI)	Crude HR (95% CI)	Adjusted‡ HR (95% CI)
All						
Disease	9,681	84.4	63.1	2.83 (2.74–2.92)	1.49 (1.42–1.56)	1.23 (1.17–1.28)
Isolation	10,936	89.7	73.4	2.30 (2.22–2.38)	1.00 (ref)	1.00 (ref)
MAC						
Disease	6,323	85.7	64.7	2.59 (2.49–2.69)	1.40 (1.32–1.49)	1.16 (1.09–1.24)
Isolation	5,756	89.5	73.2	2.27 (2.16–2.38)	1.00 (ref)	1.00 (ref)
M. xenopi						
Disease	2,263	80.2	56.8	3.49 (3.29–3.70)	1.71 (1.57–1.86)	1.39 (1.27–1.52)
Isolation	2,932	88.7	71.9	2.39 (2.23–2.55)	1.00 (ref)	1.00 (ref)
M. fortuitum						
Disease	265	84.9	64.8	2.70 (2.21–3.18)	1.47 (1.17–1.85)	1.17 (0.92–1.48)
Isolation	714	89.8	76.0	2.63 (2.27–2.99)	1.00 (ref)	1.00 (ref)
M. abscessus						
Disease	245	88.6	72.8	2.23 (1.71–2.74)	1.25 (0.87–1.80)	1.14 (0.76–1.72)
Isolation	185	91.4	77.1	2.10 (1.46–2.74)	1.00 (ref)	1.00 (ref)
M. kansasii						
Disease	158	84.2	62.3	4.37 (3.37–5.37)	0.91 (0.65–1.29)	1.15 (0.78–1.68)
Isolation	106	82.1	52.0	3.97 (2.89–5.05)	1.00 (ref)	1.00 (ref)
Other						
Disease	427	84.8	66.7	3.08 (2.62–3.55)	1.55 (1.29–1.87)	1.27 (1.04–1.55)
Isolation	1,243	92.8	77.6	1.94 (1.72–2.15)	1.00 (ref)	1.00 (ref)

*Comparisons between all registered Ontario residents with incident species-specific NTM isolation and NTM disease respectively; Matching not required for this analysis, and so adjustment was made for all covariates of interest. NTM disease was defined as >1 positive sputum for the same species or 1 positive bronchoscopic or biopsy specimen. NTM isolation was defined as 1 positive sputum sample for NTM. MAC, *Mycobacterium avium* complex; NTM, nontuberculous mycobacterium; ref, reference; SMR, standardized mortality ratio.
†Standardized by sex and age in 5-y strata using data for the Ontario population.
‡Adjusted for sex, age, income quintile, location, Adjusted Clinical Group case mix system, baseline underlying conditions (asthma, chronic obstructive pulmonary disease, diabetes, HIV infection, rheumatoid arthritis, chronic kidney disease, gastresophageal reflux disease, bronchiectasis, interstitial lung disease, cystic fibrosis, prior tuberculosis, lung cancer, solid organ transplantation or bone marrow transplantation), health use (number of hospitalizations and emergency department visits in year before index date), and NTM disease diagnosis during follow-up as time-varying covariate (for NTM isolation group only).

Table 5. Multivariable associations between baseline clinical variables and death among all patients with incident NTM pulmonary disease, Ontario, Canada, 2001–2013*

Variable	Value, n = 9,681	Adjusted† HR (95% CI)	p value
Male sex, no. (%)	49.1	1.47 (1.38–1.57)	<0.0001
Median age, y (IQR)	70 (58–78)	1.05 (1.05–1.05)	<0.0001
Income quintile,‡ %			
1 (lowest; reference)	26.7	–	–
2	21.7	0.93 (0.86–1.02)	0.1267
3	18.0	0.90 (0.82–0.99)	0.0332
4	16.3	0.86 (0.78–0.95)	0.0024
5	17.1	0.82 (0.74–0.90)	<0.0001
Residential setting,§ %			
Urban (reference)	89.5	–	–
Suburban	7.8	0.98 (0.87–1.10)	0.7091
Rural	2.7	1.04 (0.87–1.25)	0.6783
ACG number,¶ %			
0–5 (reference)	11.9	–	–
6–10	42.5	1.21 (1.05–1.39)	0.0084
≥11	45.6	1.44 (1.25–1.66)	<0.0001
Underlying condition, %			
Asthma	35.1	0.88 (0.82–0.94)	0.0003
COPD	51.3	1.38 (1.29–1.48)	<0.0001
Diabetes	19.9	1.05 (0.97–1.13)	0.2505
Rheumatoid arthritis	3.5	1.19 (1.01–1.39)	0.0339
Chronic kidney disease	8.1	1.40 (1.27–1.55)	<0.0001
GERD	17.3	0.93 (0.86–1.01)	0.0832
Bronchiectasis	14.2	0.76 (0.69–0.84)	<0.0001
Interstitial lung disease	8.1	1.51 (1.37–1.68)	<0.0001
Lung cancer	8.0	3.03 (2.78–3.32)	<0.0001
HIV infection	1.8	3.56 (2.81–4.49)	<0.0001
Cystic fibrosis	1.0	1.95 (1.37–2.77)	0.0002
Solid organ transplant	1.4	1.06 (0.81–1.38)	0.6849
Bone marrow transplant	0.6	2.77 (1.93–3.97)	<0.0001
Prior tuberculosis	1.8	0.66 (0.50–0.87)	0.0037
Hospitalizations,# mean ± SD	0.41 ± 0.93	1.09 (1.05–1.14)	<0.0001
Emergency department visits,# mean ± SD	0.93 ± 1.24	1.16 (1.13–1.20)	<0.0001
NTM species, %			
MAC (reference)	65.3	–	–
M. xenopi	23.4	1.22 (1.13–1.31)	<0.0001
M. fortuitum	2.7	1.02 (0.84–1.23)	0.8538
M. abscessus	2.5	0.98 (0.78–1.24)	0.8841
M. kansasii	1.6	1.25 (0.99–1.57)	0.0636
All other species	4.4	0.94 (0.80–1.10)	0.4306

*Multivariable Cox proportional hazards model including all 9,681 registered Ontario residents with incident NTM pulmonary disease (>1 positive sputum sample for the same species or 1 positive bronchoscopic or biopsy specimen). No matching required for this analysis, and so all covariates of interest were assessed. ACG, Adjusted Clinical Group; COPD, chronic obstructive pulmonary disease; GERD, gastresophageal reflux disease; IQR, interquartile range; MAC, *Mycobacterium avium* complex; NTM, nontuberculous mycobacteria. Dashes indicate reference level for the variable (values not calculated).
†Adjusted for sex, age, income quintile, location, ACG case mix system, baseline underlying conditions (asthma, COPD, diabetes, HIV, rheumatoid arthritis, chronic kidney disease, GERD, bronchiectasis, interstitial lung disease, cystic fibrosis, prior tuberculosis, lung cancer, solid organ transplantation or bone marrow transplantation), health use (number of hospitalizations and emergency department visits in year before index date), and NTM disease diagnosis during follow-up as time-varying covariate (for NTM isolation group only).
‡Totals do not add to 100% because of missing income data in 0.4% of patients
§Residential setting characterized by Rural Index of Ontario (*19*).
¶Number of ACG diagnoses using the ACG case mix system (*16*).
#Number of events in the year before entry.

(*3*). The significant associations that we observed between death and interstitial lung disease, lung cancer, HIV infection, cystic fibrosis, and bone marrow transplantation all seem plausible given the risks for death generally conferred by these clinical factors. Some underlying conditions undoubtedly emerged after the index date in affected patients and in unexposed controls. We elected to ignore any mortality effect of subsequently emerging underlying conditions, favoring the development of a mortality estimate based on information at the time of diagnosis.

Given the high frequency of bronchiectasis in NTM-PD (*5*), it is noteworthy that bronchiectasis in our study was uncommon, measured as 8.5% and 6.1% among matched NTM-PD and NTM-PI patients, respectively, and 14.2% and 10.5% among all NTM-PD and NTM-PI patients. Our reliance on a simple unvalidated diagnostic code definition probably failed to identify the presence of bronchiectasis in many patients. The underappreciation of bronchiectasis might have been greater among the groups of patients with NTM, wherein one would expect a high proportion of

bronchiectasis. Accordingly, assuming that bronchiectasis per se increases death, our death estimates in NTM groups might be overestimates.

Our work has several important limitations. First, because of an absence of clinical data, our definition of NTM-PD is based exclusively on microbiology, which probably misclassifies some patients with NTM colonization as having disease. Nevertheless, this misclassification rate most likely is small because microbiological-based definitions of NTM-PD exhibit high accuracy (*11*,*20*,*23*,*24*). Furthermore, the resulting effect of diagnostic misclassification would probably not significantly alter our findings. Misclassifying some patients with NTM-PI as NTM-PD would be expected to incorrectly place some patients with milder illness into the NTM-PD group and result in an attenuated apparent death attributable to NTM-PD. Therefore, the NTM-PD death rate is perhaps somewhat greater than we observed. There could be a stage migration phenomenon (*25*), by selective misclassification of the "more severe" NTM-PI as NTM-PD, apparent death could be reduced in both NTM-PI and NTM-PD. The same effect is also at play in our definition of NTM-PD with respect to the time between a first and a second positive sputum culture. Rather than arbitrarily defining a time period between a first and a second positive sputum culture, which would separate patients with "initial NTM-PI progressing to NTM-PD" and "initial NTM-PD," we defined all patients with 2 positive sputa at any time during the study period as having NTM-PD. The index date was always the date of the first positive sputum, which introduces an immortal time bias in the patients with NTM-PDd, defined as the time between the first and second positive sputum sample and leads to some underestimation of the associated mortality of NTM. Second, the lack of comprehensive medication information precludes assessing the effect of antimycobacterial treatment on survival and limits our ability to control for severity of some comorbid illnesses, such as COPD. Third, we were unable to ascertain cause of death for patients in our study. Prior studies have yielded mixed results in this area. Two studies of MAC-PD from separate NTM clinics in Japan reported that most patients died of causes other than their NTM-PD (*7*,*8*), whereas a study from 1 NTM clinic in the United States found that most deaths were NTM-related (*10*). We suspect that the higher proportion of deaths from NTM in the US study occurred because the patients were substantially younger at diagnosis, making deaths from other causes much less likely. Fourth, our propensity score matching was unsuccessful for 9% of NTM-PI patients and 13% of NTM-PD patients, and unmatched patients were older and had more underlying conditions. Although the effect of omitting these patients on our calculated HRs is not clear, an underestimation of the true risk for death is likely, in that unmatched NTM patients had substantially lower survival than did matched NTM patients. Fifth, the lack of data on patients with negative mycobacterial cultures precluded using such patients as unexposed controls. Other factors detected by clinicians and triggering a request to collect respiratory specimens for mycobacterial studies might have confounded our mortality estimates.

In summary, patients with NTM-PD have significantly lower survival than do appropriately matched population controls. This increasingly common health problem is clearly associated with not only substantial illness but with death as well. Further work should clarify the mortality effects of co-existing conditions, such as COPD, asthma, and bronchiectasis, infection with different NTM species, especially *M. xenopi*, specific antimicrobial treatment of the NTM infection, and cause of death among NTM patients.

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Variegated Squirrel Bornavirus 1 in Squirrels, Germany and the Netherlands

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We screened squirrels in Germany and the Netherlands for the novel zoonotic variegated squirrel bornavirus 1 (VSBV-1). The detection of VSBV-1 in 11 squirrels indicates a considerable risk for transmission to humans handling those animals. Therefore, squirrels in contact with humans should routinely be tested for VSBV-1.

The family *Bornaviridae* comprises the classical mammalian bornaviruses (*Mammalian 1 bornavirus* with borna disease virus; BoDV-1 and -2); avian bornaviruses (*Passeriform 1/2 bornavirus*, *Psittaciform 1/2 bornavirus*, *Waterbird 1 bornavirus*); and a recently described *Elapid 1 bornavirus* from snakes (Loveridge's garter snake virus 1) (*1*). BoDV-1 and -2 are considered nonzoonotic (*6*–*8*). The bicolored white-toothed shrew (*Crocidura leucodon*) has been proposed as a reservoir species for BoDV-1 (*2*–*5*). In addition to the exogenous viruses, endogenous bornavirus-like genomic elements have been found within the genome of humans and several animal species, including the 13-lined ground squirrel (*Spermophilus tridecemlineatus*) (*9*,*10*).

In 2015, a novel zoonotic bornavirus, the variegated squirrel bornavirus 1 (VSBV-1; new species *Mammalian 2 bornavirus*), was discovered in tissue samples from the central nervous system (CNS) of 3 persons with encephalitis, which resulted in death (*11*). The patients were breeders of variegated squirrels (*Sciurus variegatoides*), and an almost identical bornavirus sequence was detected in 1 contact squirrel kept by one of the breeders. Phylogenetic analysis classified VSBV-1 as a unique member of a new species of the genus *Bornavirus* (*11*).

We identified more VSBV-1 infected squirrels in Germany and the Netherlands. We also describe reliable molecular and serologic methods for in vivo detection of zoonotic VSBV-1 and its phylogenetic characterization.

The Study

During 2015, samples were collected from 468 squirrels representing 14 species. Sampled squirrels were from varying locations: private holdings with up to 45 animals per holding, zoological gardens, or roadkill (Table 1). Locations represented 3 countries: Germany (399 samples from 28 holdings), the Netherlands (49 samples from 4 holdings), and the United Kingdom (20 samples from roadkill). Most live squirrels were sampled at the request of the owners. At least 1 swab sample and, if possible, duplicates of oral swab samples were collected from living animals, and brain samples were tested from dead squirrels. Blood samples (EDTA/heparin) were also available from 164 squirrels (Table 1). We obtained congruent results when we tested swabs and brain samples by 2 different VSBV-1–specific quantitative reverse transcription PCR (qRT-PCR) assays (*11*). Blood samples were centrifuged, and the resulting plasma was tested for the presence of viral RNA by using qRT-PCR and for bornavirus-specific IgG by using an indirect immunofluorescence assay (*11*).

We detected VSBV-1 RNA and bornavirus-specific antibodies in 11 (2.6%) of the 468 animals. These 11 VSBV-1–positive squirrels (7 male, 4 female) belonged to the family Sciuridae, subfamilies Sciurinae (variegated squirrels, *S. variegatoides*; 6 animals) or Callosciurinae (Prevost's squirrel, *C. prevostii*; 5 animals) (Table 1). None of the animals showed clinical signs associated with CNS disorders. Of the 11 VSBV-1–positive animals, 9 were from 4 squirrel breeders in Germany (holdings I–IV). The remaining 2 were from a private holding in the Netherlands (holding V). Of the 6 variegated squirrels, 3 originated from holding I, where the first VSBV-1–positive

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Table 1. Results of testing for VSBV- in oral swab, blood, and brain samples from squirrels of 4 Sciuridae subfamilies collected in Germany, the Netherlands, and the United Kingdom, 2015*

Subfamily/species	No. positive/no. sampled	Available sample type					
		SS, B, BM	SS, BM	SS, B	BM, B	SS	BM
Callosciurinae							
Callosciurus erythraeus	0/7	0	4	2	1	0	0
Callosciurus prevostii	5/57	5	0	26	11	14	1
Tamiops mcclellandii	0/2	0	0	2	0	0	0
Tamiops swinhoei	0/4	0	0	0	0	4	0
Ratufinae							
Ratufa macroura	0/2	0	1	0	0	1	0
Sciurinae							
Sciurus aureogaster	0/5	0	0	0	0	5	0
Sciurus carolinensis	0/12	0	0	0	0	0	12
Sciurus granatensis	0/22	0	0	4	0	18	0
Sciurus niger	0/2	0	0	1	0	1	0
Sciurus variegatoides	7†/212	46	0	63	0	103	0
Sciurus vulgaris	0/127	0	2	1	0	62	62
Xerinae							
Sciurotamias davidianus	0/9	0	1	2	0	6	0
Tamias sibiricus	0/1	0	0	0	0	1	0
Tamias striatus	0/6	0	1	0	0	5	0
Total	12†/468	51	9	101	12	220	75

*Swab samples were taken from the oral cavity of each investigated animal. Oral swab samples and, if available, brain samples were tested by RT-qPCR. Blood samples (in EDTA or heparin), wherever available, were tested by quantitative reverse transcription PCR as well as by indirect immunofluorescence assay. B, blood, BM, brain material; SS, swab sample; VSBV-1, variegated squirrel bornavirus 1.
†Includes the variegated squirrel in which VSBV-1 was initially discovered (*11*).

animal was identified in 2014 (*11*), and the remaining 3 were from holdings II and III. Squirrels from the 3 holdings were traded for breeding. Of the 5 VSBV-1–positive Prevost's squirrels, 3 came from holding IV in Germany, and 2 were from holding V. The positive squirrels in the Netherlands had been transferred from a breeder in Germany in 2011–2012, but we could establish no direct epidemiologic link to the holdings in Germany where we found VSBV-1–positive animals.

All animals with detectable virus as well as 40 negative squirrels from the same holdings were euthanized and checked for macroscopic lesions. We applied hematoxylin and eosin staining to formalin-fixed, paraffin-embedded brain sections from 9 of 11 animals and performed immunohistochemistry as described previously (*12*). A BoDV-positive horse brain sample served as positive control. Histopathology showed that 5 of 9 VSBV-1–positive animals, variegated squirrels as well as Prevost's squirrels, had

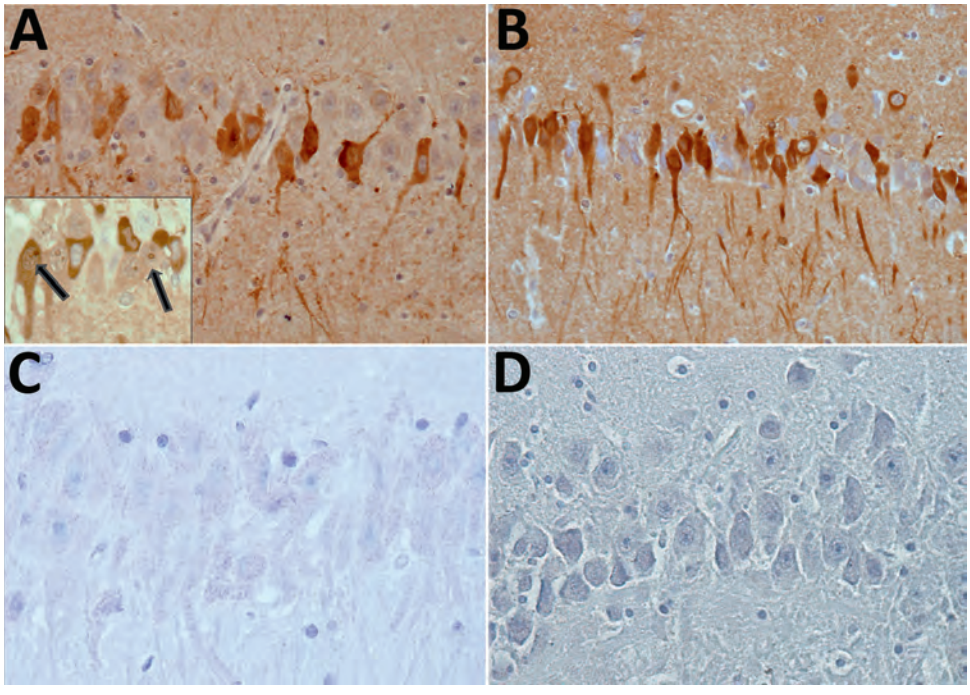

Figure 1. Immunohistochemical detection of bornavirus X protein (A) and phosphoprotein (B) in hippocampal neurons of a brain of a Prevost's squirrel (*Callosciurus prevostii*) collected in Germany in 2015. Viral antigen is shown in nuclei or cytoplasm and processes. Inset shows intranuclear dot (inclusion body) in cells with and without cytoplasmic immunostaining (arrows). No staining was observed for bornavirus X protein (C) or phosphoprotein (D) in a bornavirus-negative variegated squirrel. Original magnification × 400.

Table 2. VSBV-1 RNA levels in samples from squirrels collected in Germany and the Netherlands in 2015 that were positive for viral RNA and VSBV-1 antibodies*

Animal and sample type	Country and holding no.										
	Germany									The Netherlands	
	I	I	I	II	II	III	IV	IV	IV	V	V
Species	*Sciurus variegatoides*						*Callosciurus prevostii*				
Identity	48/15-1	48/15-2	48/15-11	49/15-1	49/15-6	75/15	122/15-1	122/15-2	133/15	3/16-1	3/16-2
Animal sex	M	F	M	M	F	M	F	M	M	F	M
EDTA blood/transudate	±	±	±	+	±	+	NA	NA	NA	±	±
Oral swab sample	+	±	++	+	+	+	+	+	+	NA	NA
Nose swab sample	±	±	+	+	±	+	+	±	±	NA	NA
Eye swab sample	+	±	+	+	±	+	NA	±	±	NA	NA
Palatine tonsil	++	++	++	++	+	++	++	++	++	+++	++
Mesenteric lymph nodes	++	++	+++	++	++	+	++	++	++	+	++
Brain	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	+++
Brachial plexus	++	++	++	++	++	++	++	++	++	+	++
Medulla oblongata	+++	+++	+++	+++	+++	+++	++	++	++	+++	+++
Trigeminal ganglion	+	+	+++	+++	++	++	NA	++	++	++	+++
Kidney	++	+	++	+++	+++	++	++	++	++	++	+++
Salivary gland	++	+	+	++	++	++	++	++	++	±	+++
Urinary bladder	++	++	+++	+++	++	+	++	++	++	++	++
Lung	++	++	+++	+++	++	++	++	+	++	±	++
Heart	++	+	++	++	++	++	+	+	++	+	++
Spleen	+	±	++	++	+	++	++	++	++	±	+++
Liver	±	±	+	+	++	+	+	+	+	±	+
Pancreas	++	+++	++	++	++	++	++	++	++	+	++
Sex organ	+	+++	+	+++	+	+++	+++	++	++	++	+++
Skeletal muscles	+	±	+	++	+	++	++	++	++	+	++
Skin	++	+	++	++	++	++	++	++	++	+	++
Nose cross-section	++	++	+++	+++	++	+++	+	++	++	++	+++

*Data are represented in VSBV-1 genome equivalent copies per milliliter of template: +++, >10^6; ++, 10^4–10^6; +, 10^2–10^4; ± 10^0–10^2). The VSBV-1–specific Mix 10 quantitative real- time PCR assay was used for quantification (limit of detection 1 genome equivalent/µL template). NA, sample not available; VSBV-1, variegated squirrel bornavirus 1.

mild nonsuppurative meningitis or encephalitis. In 8 of 9 animals, we also detected intranuclear eosinophilic Joest-Degen inclusion bodies in scattered neurons of brain, spinal cord, and trigeminal as well as spinal ganglia (data not shown). We observed bornavirus-specific phosphoprotein and X protein throughout the brain in neurons, glial cells, and in a few ependymal cells in nuclei, cytoplasm, and cellular processes (Figure 1). We tested a panel of organ samples from all euthanized animals by qRT-PCR. All animals for which swab samples were VSBV-1–positive harbored considerable VSBV-1 genome loads, whereas animals for which swab samples were negative for viral RNA were also negative for viral loads in all tested organs (Table 2; data for control animals not shown). We found the highest viral RNA loads in the CNS and organs (kidney, nose, bladder, salivary gland, and sex organs), which could play a role in viral shedding and transmission (Table 2). Skin sections were also positive, indicating that VSBV-1 has broad cell and organ tropism. We were able to cocultivate infected primary squirrel cells with a permanent cell line and to isolate infectious virus from these passaged cells.

We sequenced the VSBV-1 genome (8,786 nt; missing only the 5′ and 3′ noncoding regions) from all 11 squirrels and compared the sequences with the published VSBV-1 prototype sequence from a variegated squirrel (GenBank accession no. LN713680) (*11*). Accession numbers of the VSBV-1 sequences from this study are LT 594381–LT5943919 (European Nucleotide Archive).

Genomes of viruses detected in squirrels from holding I showed the highest similarity with 1–6 nt substitutions, resulting in 1–3 aa changes, whereas the other sequences exhibited more variability. We found ≤29 nt substitutions causing ≤19 aa changes. The mutations were evenly distributed over the coding regions, but in viral genomes of squirrels from the same holding, they often occurred at the same position (Figure 2, panel A).

We aligned the 11 VSBV-1 sequences with those from other bornaviruses by using MAFFT (Multiple Alignment using Fast Fourier Transform), and we used the best-fit model TIM2+I+G4 to construct a phylogenetic tree (IQ-Tree version 1.3.10; http://iqtree.cibiv.univie.ac.at) with 1,000 bootstrap replicates. The novel VSBV-1 genomes clustered with the 2 human- and squirrel-derived prototype sequences LN 713680 and LN71368, forming a unique VSBV-1 clade separate from other mammal, avian, and snake bornaviruses (sequence identity ≤69%; Figure 2, panel B).

Conclusions

We screened 468 squirrels and identified 11 VSBV-1–positive animals, including squirrels belonging to 2 subfamilies

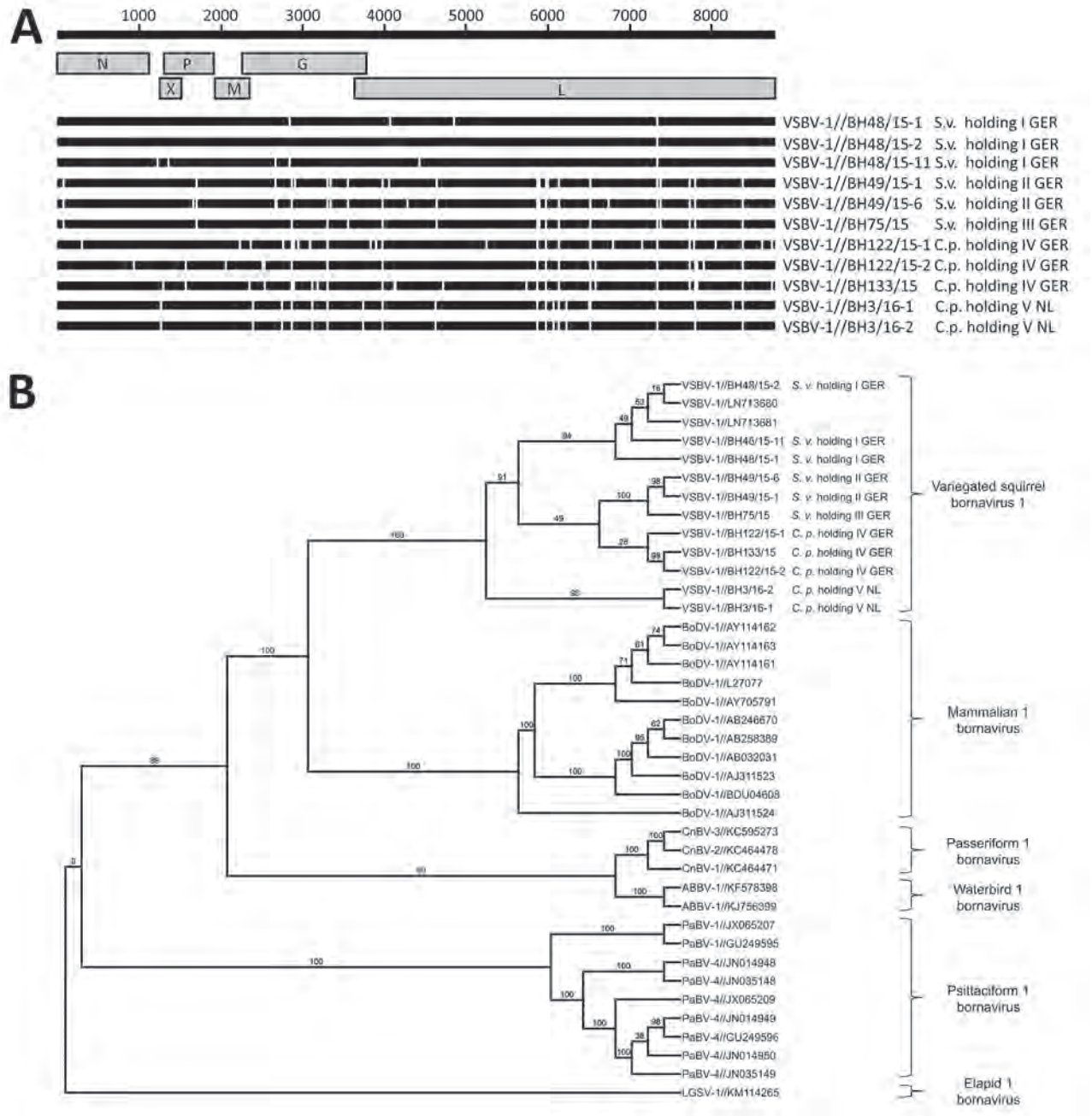

Figure 2. Analysis of 11 newly identified VSBV-1 genomes from squirrels collected in Germany and the Netherlands, 2015, in comparison with related bornaviruses. A) New sequences aligned with published squirrel-derived VSBV-1 genome (GenBank accession no. LN713680). The upper black bar indicates the reference sequence; gray boxes depict the genome. White bar sections for each animal sequence indicate nucleotide variations. The new sequences show 1–29 nucleotide differences compared to the published prototype sequence. G, glycoprotein; L, large structural protein; M, matrix; N, nucleoprotein; P, phosphoprotein; X, nonstructural protein. B) Phylogenetic tree of VSBV-1 isolates from this study (labeled) and comparison sequences. Tree was constructed using the maximum-likelihood method. Numbers along branches are bootstrap values. GER, Germany; NL, the Netherlands; VSBV-1, variegated squirrel bornavirus 1.

of the family Sciuridae. Although the VSBV-1–positive squirrels originated from different holdings and belonged to different subfamilies, the viral genome sequences formed a distinct VSBV-1 cluster. Whole-genome analyses provided no evidence for specificmutation patterns with regard to zoonotic potential or species-specific adaptations.

None of the VSBV-1-positive animals showed clinical signs of infection. Highest viral genome loads were

found in the CNS, followed by the oral cavity and skin, indicating the potential for transmission to humans through scratching or biting. Only those squirrels positive for VSBV-1 by qRT-PCR displayed bornavirus-specific antibodies. Although serologic analyses support the qRT-PCR results, collecting serum samples is difficult and often not feasible for private breeders. Our data suggest that screening of swab samples is a suitable and reliable tool for noninvasive monitoring of squirrels for VSBV-1 infection. The prevalence of 3.3% for *S. variegatoides* and 8.8% for *C. prevostii* squirrels indicates a considerable risk for transmission to humans handling those animals without taking precautionary measures. We therefore recommend routine testing of squirrels in contact with humans, such as those in breeding and holding facilities or zoological gardens, at least from the subfamilies Sciurinae and Callosciurinae.

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Genetically Diverse Filoviruses in *Rousettus* and *Eonycteris* spp. Bats, China, 2009 and 2015

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Genetically divergent filoviruses detected in *Rousettus* and *Eonycteris* spp. bats in China exhibited 61%–99% nt identity with reported filoviruses, based on partial replicase sequences, and they demonstrated lung tropism. Co-infection with 4 different filoviruses was found in 1 bat. These results demonstrate that fruit bats are key reservoirs of filoviruses.

Filoviruses (family *Filoviridae*) are nonsegmented, negative-strand RNA viruses belonging to 3 genera: *Marburgvirus*, *Ebolavirus*, and *Cuevavirus*. *Marburgvirus* comprises 1 species, *Marburg marburgvirus*, which includes Marburg virus (MARV) and Ravn virus. *Ebolavirus* comprises 5 species, *Zaire ebolavirus* (ZEBOV), *Sudan ebolavirus*, *Bundibugyo ebolavirus*, *Taï Forest ebolavirus*, and *Reston virus* (RESTV). *Cuevavirus* comprises 1 species, *Lloviu cuevavirus* (*1*). Filovirus-associated diseases, especially those caused by ZEBOV and MARV, are recognized as a major threat to public health, causing high rates of death among humans and nonhuman primates.

Bats have been implicated as natural reservoirs for filoviruses (*2*,*3*) on the basis of serologic evidence from 19 bat species in 8 countries across Asia, Africa, and Europe *(2*,*4*–*9*). In addition, filovirus RNA has been detected in 8 bat species from 7 countries in the same regions (*2*–*4*,*10*–*13*). Outbreaks of Marburg hemorrhagic fever among miners in Uganda in 2007 were traced to bat MARV (*11*). In addition, we previously discovered filovirus antibodies in several bat species in China (*14*). This finding was further confirmed by He et al., who detected filovirus RNA in brown fruit bats (*Rousettus leschenaultii*) in China (*10*). Considering the diversity of bat species in the world, long-term surveillance of bat filoviruses is essential for better understanding of distribution, diversity, and ecology of these viruses. We conducted a study to determine the diversity of filoviruses among bats in Yunnan Province, China.

The Study

We captured 150 apparently healthy adult bats from 2 caves in Yunnan Province, China: 1 in Jinghong City in November 2009, and 1 in Mengla County in December 2015 (Table 1; Figure 1). The bat species we collected were *Hipposideros armiger*, *Aselliscus stoliczkanus*, *Myotis ricketti*, *Rhinolophus Monoceros*, *Miniopterus fuscus*, *Ia io*, *Eonycteris spelaea*, and *Rousettus* sp. We humanely killed all bats and collected their hearts, intestines, lungs, spleens, kidneys, livers, brains, and blood for testing. We used 2 methods to analyze bat lung tissues for presence of filovirus RNA: first, we used nested PCR with the primers FV F1/R1 and FV F2/R2 (*10*), and next, we used quantitative PCR (qPCR) with 3 groups of qPCR with primers and probes designed from viral sequences obtained in this study (online Technical Appendix Table, https://wwwnc.cdc.gov/EID/article/23/3/16-1119-Techapp1.pdf).

Using degenerate nested PCR, we detected filovirus RNA in 15 fruit bat specimens (*E. spelaea* and *Rousettus* sp.); the specimens comprised 10 (23.3%) of 43 *E. spelaea* and *Rousettus* sp. collected in 2009 and 5 (11.9%) of 42 collected in 2015. Using qPCR, we detected filovirus RNA in 20 specimens from *E. spelaea* (n = 4) and *Rousettus* sp. (n = 16) bats: 10 (23.3%) of the bats were collected in 2009 and 10 (23.8%) in 2015. No filovirus was detected in other bat species studied (Table 1). The 310-bp L gene sequences (GenBank accession nos. KX371873–KX371890) exhibited 65%–99% nt identity among themselves and 61%–99% nt identity with known filoviruses. Phylogenetic analysis showed that the sequences from the bats formed 3 independent groups, groups 1–3. Groups 1 and 2 comprised 6 and 11 sequences, respectively, all of which were obtained in this study (Figure 2). Group 3 comprised 2 sequences, 1 from this study and 1 previously published (*10*). Pairwise distance analysis indicated that sequences in group 1 share the highest nucleotide identity (75%–78%) with MARV and those in group 2 share the highest identity (69%–74%) with Ravn virus. The 2 sequences in group 3 are highly similar and share 66%–70% nt identity with other filovirus species. Of note, 1 bat specimen (no. 9447) was co-infected

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Table 1. Filovirus infection detected in bat samples by PCR, ELISA, and Western blot, Yunnan Province, China, 2009 and 2015*

Bat species, by year and month of collection/location	No. positive/no. tested (%)					
		Quantitative	ELISA†		Western blot†	
	RT-PCR	PCR	ZEBOV	RESTV	ZEBOV	RESTV
2009 Nov/Jinghong City						
Hipposideros armiger	0/15	0/15	0/15	0/15	0/15	0/15
Rhinolophus monoceros	0/4	0/4	0/4	0/4	0/4	0/4
Ia io	0/3	0/3	0/3	0/3	0/3	0/3
Miniopterus fuscus	0/1	0/1	0/1	0/1	0/1	0/1
Myotis ricketti	0/27	0/27	1/27 (3.7)	1/27 (3.7)	1/27 (3.7)	1/27 (3.7)
Eonycteris spelaea and *Rousettus* sp.	10/43 (23.3)	10/43 (23.3)	5/43 (11.6)	6/43 (13.9)	2/43 (4.6)	2/43 (4.6)
2015 Dec/Mengla County						
Aselliscus stoliczkanus	0/15	0/15	0/15	0/15	0/15	0/15
E. spelaea and *Rousettus* sp.	5/42 (11.9)	10/42 (23.8)	14/25 (56)	7/25 (28)	11/25 (44)	4/25 (16)

*EBOV, *Zaire ebolavirus*; RESTV, *Reston virus*.
†ELISA and Western blot results for samples collected in 2009 were from a previous study (*14*).

with 4 different filovirus strains (BtFiloYN9447–1 to 9447–4) with high divergence (Figure 2; online Technical Appendix Figure 1). To further determine the phylogenetic relationship of these viruses with known filoviruses, we amplified more L gene sequence (1,475 bp) for strains BtFiloYN2162 and BtFiloYN9447–1. Similar to the 310-bp sequences, the 1,475-bp sequence of the BtfiloYN2162 shared 99% identity with BtDH04 at the nucleotide level, the 1,475-bp sequence of BtFiloYN9447–1 shared 62%–71% with known filoviruses.

To determine the tissue tropism of these viruses, we performed qPCR with primers and probes designed for each of the 3 different groups (online Technical Appendix Table). Results showed that filoviruses were mainly located in the lung and that genome copy numbers ranged from 29 to 523,582/mg of tissue (Table 2). Only 2 bat blood samples (nos. 2202 and 9447) were positive for filovirus RNA; 5 samples (nos. 2202, 2188, 9434, 9442, and 9447) contained filoviruses with more widespread tissue tropism. We were unable to isolate virus from PCR-positive samples by using Vero-E6 cells.

To detect filovirus IgG and IgM, we expressed His-tagged truncated nucleoproteins from RESTV or ZEBOV in *Escherichia coli* and used them as antigens (online

Figure 1. Bat collection sites for a study on genetically diverse filoviruses in *Rousettus* and *Eonycteris* spp. bats in China. Triangles indicate Jinghong City and Mengla County, Yunnan Province, where 150 apparently healthy adult bats were collected from 2 caves in November 2009 (Jinghong City) and December 2015 (Mengla County). Inset map shows the location of Yunnan Province in China.

Table 2. Virus tropism and quantification in different tissues of *Eonycteris spelaea* and *Rousettus* sp. bats, China, 2009 and 2015

Sample no.	Species	Sex	Primer group*	Positive organs (viral genome copies/mg tissue or viral genome copies/μL blood)
2162	*E. spelaea*	M	3	Lung (119)
2176	*Rousettus* sp.	M	2	Lung (2,103)
2180	*Rousettus* sp.	F	2	Lung (42)
2181	*Rousettus* sp.	M	2	Lung (202)
2187	*Rousettus* sp.	F	1	Lung (46)
2188	*Rousettus* sp.	F	1	Lung (43), liver (400), kidney (42)
2190	*Rousettus* sp.	M	2	Lung (195)
2196	*Rousettus* sp.	F	1	Lung (123)
2199	*Rousettus* sp.	F	1	Lung (74)
2202	*Rousettus* sp.	F	1	Lung (864), liver (368), kidney (342), intestine (254), heart (807), blood (1)
9428	*Rousettus* sp.	F	2	Lung (156)
9434	*Rousettus* sp.	F	1	Lung (554), spleen (3,014), kidney (380), heart (320), intestine (2751)
9435	*Rousettus* sp.	F	2	Lung (127)
9442	*E. spelaea*	M	2	Lung (180), spleen (88)
9445	*Rousettus* sp.	M	1	Lung (143)
9447	*Rousettus* sp.	F	2	Lung (132), liver (154), spleen (661)
9447	Rousettus sp.	F	1	Lung (106,606), liver (220,051), spleen (523,582), kidney (41,653), brain (4,885), heart (17,982), intestine (11,788), blood (485)
9454	*Rousettus* sp.	M	1	Lung (448)
9457	*E. spelaea*	M	1	Lung (52)
9459	*Rousettus* sp.	F	2	Lung (182)
9463	*E. spelaea*	M	2	Lung (114)

*These represent primers and probes designed based on partial sequences of the virus L gene obtained in this study. Sequence information is provided in the online Technical Appendix (https://wwwnc.cdc.gov/EID/article/23/3/16-1119-Techapp1.pdf).

Technical Appendix). In this experiment, we used 25 bat samples from 2015 that had enough serum volume for testing; 14 samples showed a strong cross-reaction with the ZEBOV nucleoprotein, and among them, 7 showed a weak cross-reaction with RESTV nucleoprotein. We used Western blotting to confirm these results; 11 of the 25 samples were positive for ZEBOV nucleoprotein and 4 for RESTV nucleoprotein (Table 1; online Technical Appendix Figure 2). No samples overlapped between those identified as positive by PCR and those identified as positive by serologic testing. Results of a serum neutralization assay with HIV pseudovirus carrying the ZEBOV glycoprotein showed that the ELISA-positive samples had no cross-neutralization activity to the pseudovirus (*14*).

Conclusions

We detected novel filovirus sequences with high divergence in *E. spelaea* and *Rousettus* sp. bats in China. Phylogenetic analysis of partial sequences suggested that at least 3 distinct groups of filovirus are circulating in fruit bats in China. The distances between these sequences indicates that the 3 groups may represent 3 novel species or genera. Of interest, we detected antibodies reacting more strongly to ZEBOV than RESTV nucleoprotein in some filovirus RNA–negative samples, suggesting that the bats were infected with another/other filovirus(es) cross-reactive with ZEBOV nucleoprotein or that nucleoproteins of the novel filoviruses were cross-reactive with ZEBOV and RESTV nucleoproteins.

The bat samples in this study were collected from 2 caves in 2009 and 2015, respectively; the caves are ≈200 km metric apart. Across the 2 different years and locations, we detected closely related viruses and found 1 bat that was acutely co-infected by 4 different filoviruses; this finding suggests that these viruses have been circulating in the 2 bat species and that densely populated bat caves provide opportunity for cross-infection with different viruses. However, considering the migration ability of the fruit bat, we cannot exclude the possibility that there are exchanges of virus between the bats in these two caves. Longitudinal surveillance with tracking tags may help to better understand the spatial–temporal distribution of these viruses in bat populations.

In previous reports, filoviruses were primarily detected in liver and spleen tissues (*4*,*15*). In our study, we primarily detected filoviruses in the lung. We suspect that lung tissues are the major target for these bat filoviruses. Thus, these filoviruses may have the potential to be transmitted through the respiratory tract.

These results will be helpful in providing a better understanding of the distribution and diversity of filoviruses, which may have implications for public health. Considering their feeding habitats, fruit bats are often in close contact with domestic animals and human populations. It is therefore necessary to establish long-term and proactive surveillance of these viruses and related diseases.

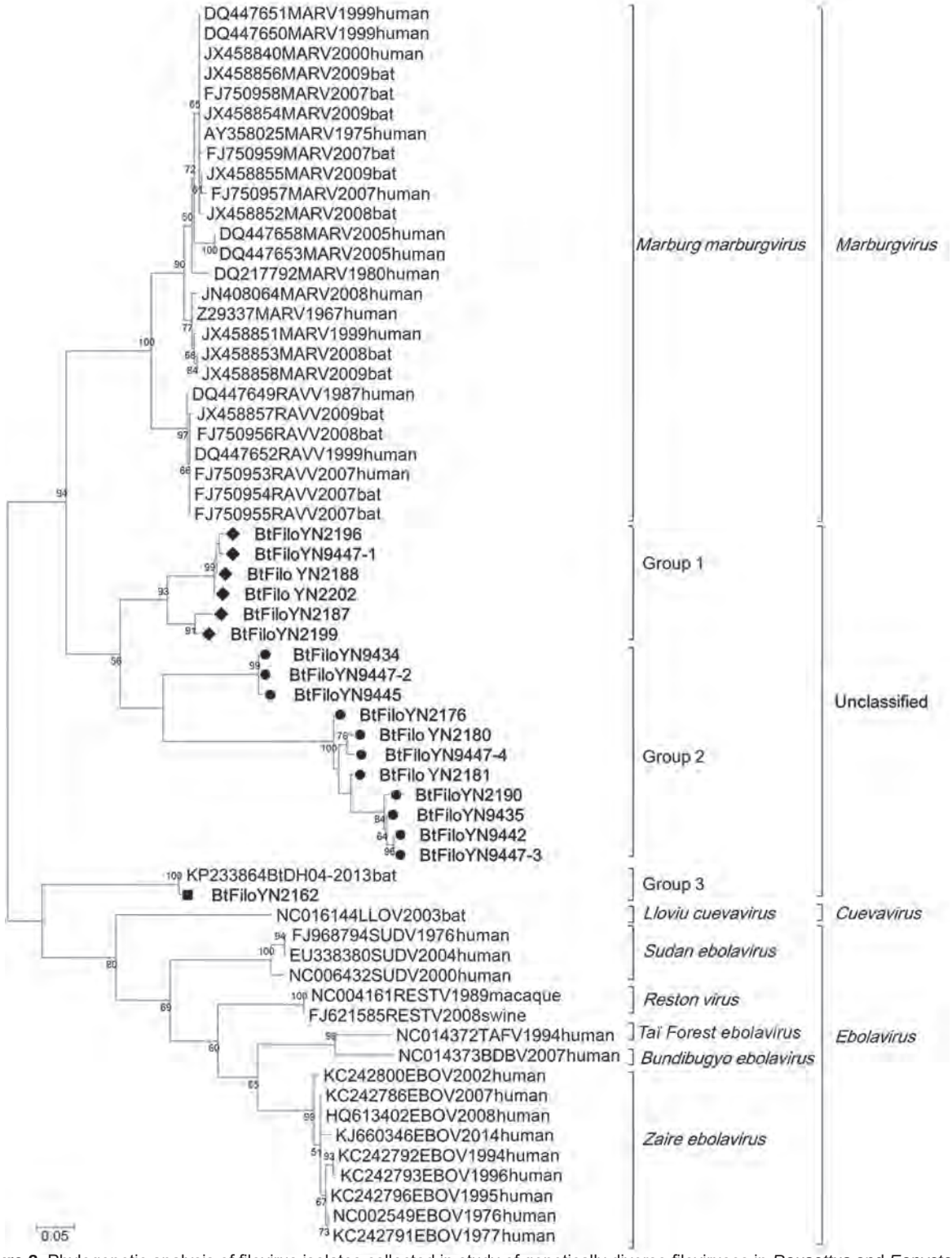

Figure 2. Phylogenetic analysis of filovirus isolates collected in study of genetically diverse filoviruses in *Rousettus* and *Eonycteris* spp. bats in China, compared with reference isolates. Analysis was based on a 310-bp segment of the filovirus L gene. Bootstrap values lower than 50 are not shown. The maximum-likelihood tree was constructed based on the 310-bp segment with 1,000 bootstrap replicates. The sequences obtained in this study are marked with a triangle (group 1), black dot (group 2), or rectangle [group 3). Sequences from GenBank are listed by their accession numbers, followed by the virus name, collection year, and host. Scale bar indicates nucleotide substitutions per site.

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Molecular, Spatial, and Field Epidemiology Suggesting TB Transmission in Community, Not Hospital, Gaborone, Botswana

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During 2012–2015, 10 of 24 patients infected with matching genotypes of *Mycobacterium tuberculosis* received care at the same hospital in Gaborone, Botswana. Nosocomial transmission was initially suspected, but we discovered plausible sites of community transmission for 20 (95%) of 21 interviewed patients. Active case-finding at these sites could halt ongoing transmission.

Tuberculosis (TB) results from rapid progression of a recently acquired *Mycobacterium tuberculosis* infection or from reactivation of a remote infection (*1*). It is critical that recent *M. tuberculosis* infections be identified because TB is more likely to develop in persons with recent infections (*2*). Furthermore, the finding of recently infected persons suggests ongoing transmission of TB, which can be interrupted by prompt identification and treatment of undiagnosed cases (*3*). However, finding undiagnosed cases remains a challenge (*4*). Name-based contact investigations have traditionally been used for this purpose, but such investigations are resource-intensive (*5*), making them less practical in countries to which TB is endemic. Genotyping of *M. tuberculosis* has emerged as a complementary method to detect ongoing transmission because persons who have the same TB genotype may be involved in the same chain of transmission (*6*). Although this assumption is relatively reliable in low-incidence countries, it is yet to be determined whether genotyping in TB-endemic settings can similarly detect ongoing transmission.

We investigated a TB cluster of 24 patients with matching *M. tuberculosis* genotypes in Gaborone, Botswana, a city with a high number of TB cases (*7*). Because almost half of these patients received care at the same hospital, nosocomial transmission was suspected. We conducted an investigation to determine if TB transmission occurred among these patients within the hospital and to identify possible alternate sites of ongoing transmission of this TB strain.

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The Study

During August 2012–January 2015, all consenting persons with TB at 26 facilities in Gaborone provided sputum samples for culture as part of the Kopanyo study (*8*). *M. tuberculosis* isolates were genotyped by 24-locus mycobacterial interspersed repetitive units–variable number tandem repeats (*9*).

We assessed nosocomial transmission by reviewing dates of hospital visits for overlap among the 24 TB cluster-associated patients. The hospital's electronic billing system was used to obtain all previous dates of admission, discharge, and visits to the accidents and emergency department that had occurred for these patients since 2004.

We interviewed each patient, using an investigation form (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/23/3/16-1183-Techapp1.pdf), to learn their primary residence; contacts; places of work and worship; and other frequented locations, including bars and combi (minibus) routes used in the 6 months before diagnosis. Primary residences of patients were mapped by using global positioning system coordinates (*8*). Ethical approval was obtained from the University of Pennsylvania, US Centers for Disease Control and Prevention, Botswana Ministry of Health, and University of Botswana.

We looked for epidemiologic links that might suggest ongoing transmission. An epidemiologic link was defined for patients having at least 1 of the following associations: overlapping visits at the hospital, living within 1 km of another cluster-associated patient (spatial link), frequenting the same locations as another cluster-associated patient, and naming another cluster-associated patient as a contact.

During the study, ≈60% of *M. tuberculosis* isolates from reported TB patients in Gaborone were genotyped. The cluster discussed in this report includes 24 (2.3%) of 1,033 total genotyped cases from Gaborone.

All patients had pulmonary disease involvement (Table 1). Ten (42%) had received care at the hospital since 2004;

[1]Other members of the Kopanyo Study Group are listed at the end of this article.

Table 1. Patient and disease characteristics in a tuberculosis cluster, Gaborone, Botswana, 2012–2015

Characteristic	No. (%)*
Patient	
Sex	
M	14 (58)
F	10 (42)
Age, median y (range)	31 (15–55)
Primary residence in Gaborone	20 (83)
Alcohol use	4 (17)
Tobacco use	3 (13)
Cough	23 (96)
History of visit to the hospital	10 (42)
Died	1 (4)
Disease-associated	
Pulmonary	24 (100)
Extrapulmonary involvement†	2 (8)
Positive sputum smear at diagnosis	12 (75)‡
HIV infection	16 (67)
CD4 cells/mL, median (range)§	310 (14–700)
Receiving antiretroviral therapy at time of tuberculosis diagnosis	9 (56)

*Data are no. (%) for 24 patients except as indicated.
†In addition to pulmonary tuberculosis, 2 patients also had extrapulmonary involvement (pleural and abdominal tuberculosis).
‡Sixteen patients had a sputum smear tested at diagnosis.
§CD4 counts were available for 11 of 16 patients with HIV infection.

most visits occurred after October 2013 (Figure 1). Except for visits by 2 patients, no patients' visits overlapped at the hospital. Patients V and X overlapped in the hospital for 3 days, albeit in separate buildings. Patient V was admitted to the hospital with a known diagnosis of TB and had started TB therapy the day before admission. Patient X was in the hospital for a week but did not start TB therapy until 13 days after patient V was admitted. No members of this cluster were healthcare workers.

Twenty (83%) patients had a primary residence in Gaborone; 14 resided in 4 distinct neighborhoods (Figure 2, panel A). The spatial link definition (residing <1 km from another cluster-associated patient) was met by 13 (54%) patients. One patient (B) lived slightly >1 km from the nearest patient (Figure 2, panel B). Two of 4 spatially linked patients (C, D, I, and W) (Figure 2, panel B) did not name each other during enrollment when asked about contacts; when interviewed again as part of this investigation, these patients confirmed spending time together around the time of their diagnosis.

One patient died and 2 were lost to follow-up. Thus, 21 patients contributed to the remaining epidemiologic links obtained from interviews (Table 2). The median number of specific locations reported by patients was 6 (range 1–13). Of the 21 patients, 11 (52%) reported attending the same bar as another patient; 1 of these patients worked as a bartender. Site visits to 2 of the bars revealed an environment conducive to TB transmission (closed, poorly ventilated space crowded with patrons and employees). Similar crowded conditions were observed in combis, and 16 (76%) patients reported using the same combi route as another patient. Eight (38%) patients attended the same church as another patient, and 6 (29%) patients named each other as a contact, suggesting transmission could have occurred among them.

Conclusions

TB genotyping surveillance prompted a targeted investigation, which, when combined with spatial and field epidemiologic data, identified unsuspected locations of possible transmission. TB transmission from 1 cluster-associated patient to another at the hospital seemed unlikely. Although 2 patients' hospital stays overlapped by 3 days, nosocomial transmission between them probably did not occur because the patients were hospitalized in separate buildings and the time from TB exposure and treatment initiation (13 days) is an extremely short time for disease to develop (*10*). Instead, the combination of links among patients suggests ongoing community transmission. Plausible sites of transmission included specific neighborhoods, bars, combi routes, and churches, transmission locations consistent with reports from other TB-endemic settings (*11*,*12*). These findings demonstrate the critical role that nonhousehold-based TB transmission plays in sub-Saharan Africa and highlight the need for identifying community-based TB transmission. A multidisciplinary approach (i.e., use of genotyping, spatial analyses, and interviews) provided us with locations where additional persons may be at risk for TB.

Our study has limitations. First, because samples from every TB case in Gaborone were not genotyped and interviews were not conducted with all persons in this cluster, key linkages between patients may have been missed.

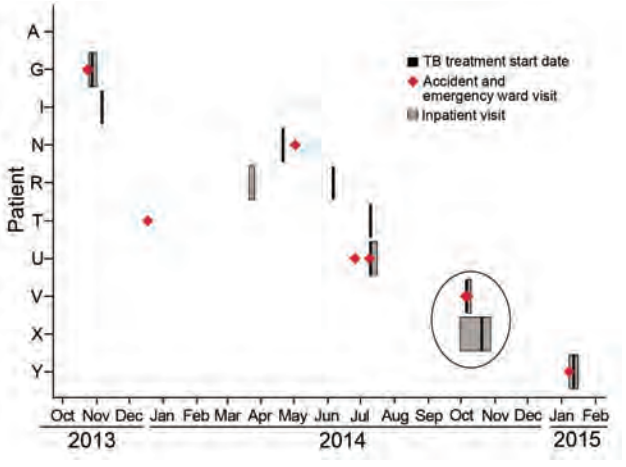

Figure 1. Timing of hospital visits and treatment for 10 tuberculosis (TB) cluster–associated patients, Gaborone, Botswana, 2013–2015. Patients were hospitalized or seen in the accident and emergency ward, and all had a history of such visits since 2004. Visits prior to October 2013 are not shown; these include visits in 2012 by patients A and I and additional visits by patients N, T, and U. None of the pre-October 2013 visits overlapped with those of other TB cluster–associated patients.

Figure 2. Residence-associated data for patients in a tuberculosis cluster, Gaborone, Botswana, 2012–2015. A) Primary residences of 20 patients are indicated by red dots. Inset map shows location of Gaborone in Botswana. Black lines demarcate neighborhoods; gray lines demarcate property parcels; pink circles represent 0.5-km radius around a patient's residence; and red rectangles indicate presence of 14 patients in 4 distinct neighborhoods, 13 of whom had spatial links. Four patients who are not depicted on this map lived outside of Gaborone and did not have any spatial links between them. B) Primary residences of 5 patients who lived in the same neighborhood. Parcels locations were intentionally not shown to protect individual case anonymity. Geodata were sourced from Statistics Botswana (http://www.cso.gov.bw).

Second, underreporting of locations might have occurred due to patients' inability to remember all locations visited; thus, some less frequented places where transmission might have occurred may have been missed. Third, we could not prove that patients who attended the same location interacted with each other at that location while infectious. Fourth, if a cluster-associated patient went to the hospital as a visitor, not a patient, their overlap with another cluster-associated patient could have been missed. Fifth, the extent to which the hospital infection-control program influenced our findings is unknown. Sixth, because 24-locus mycobacterial interspersed repetitive units–variable number tandem repeats characterizes only a portion of the *M. tuberculosis* genome, it is possible for 2 different strains to appear similar. Whole-genome sequencing could help confirm (or refute) the findings in this investigation.

Although genotyping is an imperfect tool for confirming transmission between patients, we know that numerous

Table 2. Epidemiologic links between patients in a tuberculosis cluster, Gaborone, Botswana, 2012–2015

Link	No./no. total	%
Location		
Any	20/21*	95
Hospital A	2/24†	8
Combi routes	16/21	76
Spatial	13/24	54
Bars	11/21	52
Churches	8/21	38
Named contacts	6/21	29
No. links		
≥2	14/21	67
≥3	11/21	52
≥4	3/21	14

*Only 21/24 cluster-associated patients were reachable for interview regarding epidemiologic links associated with contacts, combi routes used, and places of socialization and worship.
†Hospital visits for 2 patients overlapped, but tuberculosis transmission between them probably did not occur because the patients were hospitalized in separate buildings and the time between tuberculosis exposure and treatment initiation (13 d) is an extremely short time for disease to develop.

patients in this cluster visited the same community locations while they were potentially infectious, which alone could justify further active case-finding at these locations. With an estimated incidence of 385 TB cases/100,000 persons in Botswana (*7*), such clues are needed to focus TB control efforts. Active case-finding targeted at the most frequently visited community locations could help stop ongoing transmission of this strain.

Members of the Kopanyo Study Group: Joyce Basotli, Ebi Bile, Rosanna Boyd, Victoria Cowger, Mbatshi Dima, Sambayawo Gwebe-Nyirenda, Cynthia Caiphus, Thandi Katlholo, Christopher Serumola, Tsaone Tamuhla, Goitseone Thamae, Onani Zimba, Pilara Khumongwana, Kitso Ramogale, Sanghuk Shin, James Shepherd.

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pncA Gene Mutations Associated with Pyrazinamide Resistance in Drug-Resistant Tuberculosis, South Africa and Georgia

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Although pyrazinamide is commonly used for tuberculosis treatment, drug-susceptibility testing is not routinely available. We found polymorphisms in the *pncA* gene for 70% of multidrug-resistant and 96% of extensively drug-resistant *Mycobacterium tuberculosis* isolates from South Africa and Georgia. Assessment of pyrazinamide susceptibility may be prudent before using it in regimens for drug-resistant tuberculosis.

Drug-resistant tuberculosis (TB) poses a significant threat to global health, with an estimated 480,000 new cases of multidrug-resistant tuberculosis (MDR TB) in 2014; 10% of these cases were classified as extensively drug-resistant tuberculosis (XDR TB) (*1*). MDR and XDR TB are associated with high mortality rates because of limited treatment options (*2*,*3*). Drug-susceptibility testing (DST) is critical for constructing MDR and XDR TB treatment regimens.

Pyrazinamide is a critical component of first-line TB regimens but is also recommended for use in drug-resistant TB regimens (*4*). Despite widespread use, phenotypic DST for pyrazinamide is not routinely performed because of the precise acidic conditions required (*5*). However, acidic environments also inhibit the growth of *Mycobacterium tuberculosis*, making phenotypic pyrazinamide DST challenging even in sophisticated TB laboratories. The need for pyrazinamide DST is underscored by the potential synergy between pyrazinamide and new TB drugs under study for treatment of drug-susceptible and drug-resistant TB (*6*).

The development of rapid molecular tests has simplified testing for drug resistance. Assays detect resistance-conferring mutations in genes associated with phenotypic resistance to isoniazid, rifampin, fluoroquinolones, ethambutol, aminoglycosides, and capreomycin. Genotypic testing for pyrazinamide may provide a simpler method for assessing drug susceptibility (*7*). Pyrazinamide resistance arises through genetic mutations in the *pncA* gene (*8*). *pncA* encodes pyrazinamidase, which converts pyrazinamide into pyrazinoic acid for its antimycobacterial activity. However, data on the frequency and diversity of *pncA* mutations in clinical settings are limited.

We characterized the frequency and diversity of polymorphisms in the *pncA* gene and estimated the prevalence of pyrazinamide resistance among patients with MDR and XDR TB in South Africa and the country of Georgia. Ethics approval for the study was obtained from Emory University, Albert Einstein College of Medicine, National Center for Tuberculosis and Lung Diseases, University of KwaZulu-Natal, and the Centers for Disease Control and Prevention.

The Study

We performed a cross-sectional study examining *pncA* polymorphisms in *M. tuberculosis* isolates from a convenience sample of patients with MDR or XDR TB who were prospectively enrolled in studies from KwaZulu-Natal, South Africa (n = 451, diagnosed 2011–2014), and Georgia (n = 103, diagnosed November 2011–April 2012) (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/23/3/16-1034-Techapp1.pdf). Cultures and DST were performed at the provincial TB reference laboratory in Durban, South Africa, and the National Reference Laboratory in Tbilisi, Georgia. Samples underwent PCR amplification followed by standard capillary sequencing of the *pncA* promoter and coding DNA sequence at the Public Health Research Institute in Newark, New Jersey, USA, as previously described (*9*). Polymorphisms were identified by alignment of nucleotide sequences to the H37Rv reference strain by using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). We calculated the frequency of each *pncA*

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Table 1. *pncA* gene sequencing and RFLP results of 74 MDR TB and 377 XDR TB isolates from KwaZulu-Natal Province, South Africa*

Mutation	*pncA* mutation type†	Frequency (no. isolates)	RFLP‡
Mutations common to MDR TB and XDR TB isolates			
1	Ins C after 456	271 (MDR TB: 7; XDR TB: 264)	MDR TB: HP XDR TB: HP (263), mixed (1)
2	TTG(L) 151 TCG(S)§	19 (MDR TB: 8; XDR TB: 11)	MDR TB: GY XDR TB: GY
3	AGG(R) 154 GGG(G)	17 (MDR TB: 3; XDR TB: 14)	MDR TB: AH XDR TB: AH
4	GCG(A) 102 GTG(V)§	15 (MDR TB: 13; XDR TB: 2)	MDR TB: BW (1), KO (12) XDR TB: BM (1), KO (1)
5	CAT(H) 71 TAT(Y)§	13 (MDR TB: 3; XDR TB: 10)	MDR TB: BH (1), BW (2) XDR TB: BH (1), BW (8), HP (1)
6	Ins A after 408§	8 (MDR TB: 1; XDR TB: 7)	MDR TB: CC XDR TB: CC (6), GY (1)
7	TCC(S) 59 CCC(P)§	7 (MDR TB: 4; XDR TB: 3)	MDR TB: BF XDR TB: BF
8	GGT(G) 132 GCT(A)§	4 (MDR TB: 1; XDR TB: 3)	MDR TB: HP XDR TB: HP
9	GGT(G) 97 GAT(D)§	2 (MDR TB: 1; XDR TB: 1)	MDR TB: KM XDR TB: M
Mutations specific to MDR TB isolates			
10	CTG(L) 35 CTA(L)¶	1	W
11	CTG(L) 35 CTA(L)¶ TTG(L) 151 TCG(S)	1	GY
12	TTC(F) 58 TCC(S)§	1	BH
13	ACT(T) 76 ATT(I)§	1	CC
14	GAG(E) 91 CAG(Q)	1	BH
15	GGT(G) 97 TGT(C)§	1	H
16	Ins A after 407§	1	CC
17	Del CAGGGTGC at 459	1	W
18	Del T at 515§	1	GO
Mutations specific to XDR TB isolates			
19	insG after 515	23	MH
20	TAC(Y) 34 GAC(D)§	5	BH
21	GTG(V) 139 GGG(G)§	3	HP
22	GTG(V) 130 GCG(A)#	2	W
23	Del G at 385, TTG(L) 151 TCG(S)	2	GY
24	GAG(E) 15 GGG(G)	1	W
25	ACC(T) 47 ATC(I)	1	KR
26	CAC(H) 51 CCC(P) §	1	HP
27	TCC(S) 65 TCT(S)¶,#	1	GD
28	TGC(C) 104 CGC(R)	1	MH
29	ACC(T) 153 CAC(H)§	1	HP
30	AGG(R) 154 TGG(W)§	1	HP
31	Ins G after 312	1	HP
32	Ins G after 313	1	HP
33	Del T at 389	1	HP
34	Ins C after 456, CTG(L) 35 CTA(L)¶	1	HP
Wild-type		40 (MDR TB: 24; XDR TB: 16)	MDR TB: BE (2), BH (4), BM (1), BW (1), CC (2), FO (1), GO (1), GY (1), HZ (1), MH (2), W (8); XDR TB: AH (1), BM (1), BW (1), CC (1), GY (1), HP (2), MH (4), mixed (1), W (4)

*del, deletion; ins, insertion; MDR, multidrug resistant; RFLP, restriction fragment-length polymorphism; SNP, single-nucleotide polymorphism; TB, tuberculosis; XDR, extensively drug-resistant. Row colors: pink, frameshift mutations (insertions or deletions); green, synonymous mutations or SNPs reported as being associated with phenotypic susceptibility to pyrazinamide; gray, SNPs reported as being associated with phenotypic resistance to pyrazinamide; blue, single-nucleotide polymorphisms not previously reported in the literature.
†Insertions and deletions are presented with the nucleotide position where the polymorphism occurred; SNPs are presented with the codon position and the original and mutated 3 nucleotides and amino acid.
‡The nomenclature used for classifying IS6110 RFLP patterns was as follows: 2 isolates with an identical *IS*6110 banding pattern were assigned the same arbitrary 1- or 2-letter code (e.g., W, HP, or AB), which started with the first observed cluster, strain A (several decades ago). *IS*6110 patterns that were similar but not identical were denoted by the addition of a number (e.g., BE1, W4, or HP81). This table shows only the letter designations; numbers have been omitted for simplicity. Strains within each of the 2-letter designations had similar RFLP patterns.
§Reported in the literature as being associated with resistance to pyrazinamide.
¶Synonymous mutation.
#Reported in the literature as being associated with susceptibility to pyrazinamide.

polymorphism and classified mutations as synonymous or nonsynonymous. We compared polymorphisms with those reported in the literature to identify any that are known to be associated with phenotypic susceptibility and to determine the proportion that are likely to confer phenotypic resistance (*10*–*15*).

To determine the effect of clonal expansion of MDR and XDR TB on the diversity of *pncA* mutations, we also compared IS*6110*-based restriction fragment-length polymorphism (RFLP) patterns with *pncA* mutations for the isolates from South Africa (conducted at the Public Health Research Institute). The distribution of RFLP patterns among isolates with identical *pncA* mutations was examined to determine if the *pncA* mutation arose de novo or may have been transmitted.

We completed targeted *pncA* gene sequencing for 554 unique patient-isolates [1 isolate/patient], 167 MDR TB and 387 XDR TB; of these, 99 (59%) of MDR TB and 215 (56%) of XDR TB patients had previously received treatment for TB. A *pncA* polymorphism was found in 117 (70%) MDR TB and 370 (96%) XDR TB isolates (Tables 1, 2). The proportion of MDR TB and XDR TB isolates with *pncA* polymorphisms did not differ significantly between those from South Africa and Georgia (MDR, 68% vs. 72%, p = 0.74; XDR, 96% vs. 90%, p = 0.73, respectively).

Table 2. *pncA* gene sequencing results of 93 MDR TB and 10 XDR TB isolates from Georgia*

Mutation	*pncA* mutation type†	Frequency (no. isolates)
Mutations specific to MDR TB isolates		
1	CAG(Q) 141 CCG(P)‡	20
2	CAT(H) 71 CGT(R)‡	8
3	CAT(H) 71 CCT(P)‡	6
4	Ins C after 420, 421ΔA, CAG(Q) 141 CCG(P)‡	3
5	TGG(W) 119 TTG(L)‡	3
6	ATC(I) 6 CTC(L)§	2
7	GAC(D) 49 GGC(G)‡	2
8	CCA(P) 69 CGA(R)‡	2
9	GTG(V) 155 GCG(A)§	2
10	TTG(L) 4 TGG(W)‡	1
11	ATC(I) 5 ACC(T)§	1
12	CTG(L) 27 CCG(P)‡	1
13	GAC(D) 49 GAG(E)‡	1
14	CAC(H) 51 TAC(Y)‡	1
15	CCG(P) 54 CAG(Q)‡	1
16	GAC(D) 63 GCC(A)‡	1
17	TCG(S) 66 CCG(P)‡	1
18	CAT(H) 71 CCT(P); Ins G after 547 (after G in GTT)	1
19	GGT(G) 97 AGT(S)‡	1
20	TAC(Y) 103 TAA(Ter)‡	1
21	TAC(Y) 103 TAG(Ter)‡	1
22	GCC(A) 134 CCC(P)	1
23	ACG(T) 142 ATG(M)‡	1
24	GGT(G) 162 TGT(C)	1
25	TGA(Ter) 187 CGA(R)§	1
26	Del A at 298	1
27	Ins A after 389 (after T in GTG)	1
28	Ins G after 449 (after sec G in GGC)	1
Wild type		26
Mutations specific to XDR TB isolates		
1	GTC(V) 7 GCC(A)‡	2
2	ACC(T) 47 AGC(S)‡	2
3	Ins TCT after 40 (after T In TGC)	1
4	GGC(G) 78 GAC(D)‡	1
5	GGT(G) 97 CGT(R)‡	1
6	TAC(Y) 103 GAC(D)‡	1
7	GGC(G) 105 CGC(R)	1
Wild type		1

*del, deletion; ins, insertion; MDR, multidrug resistant; SNP, single-nucleotide polymorphism; TB, tuberculosis; XDR, extensively drug resistant. Row colors: pink, frameshift mutations: insertions or deletions; green, synonymous mutations or SNPs reported as being associated with phenotypic susceptibility to pyrazinamide; gray, SNPs reported as being associated with phenotypic resistance to pyrazinamide; blue, SNPs not previously reported in the literature.
†Insertion and deletions are presented with the nucleotide position where the polymorphism occurred; single nucleotide polymorphisms are presented with the codon position and the original and mutated three nucleotides and amino acid
‡Reported in the literature as being associated with resistance to pyrazinamide.
§Reported in the literature as being associated with susceptibility to pyrazinamide.

A total of 69 distinct *pncA* polymorphisms were identified (Tables 1 and 2). Of these, 12 were insertions (313 patient-isolates), 5 were deletions (6 patient-isolates), and 52 were single-nucleotide polymorphisms (SNPs; 168 patient-isolates); all but 2 of these were nonsynonymous. No polymorphism was found in common between the isolates from South Africa and Georgia. Among the *pncA* SNPs identified, only 6 (9 patient-isolates) have previously been associated with phenotypic pyrazinamide susceptibility (*10*–*12*); 40 SNPs have been associated with phenotypic pyrazinamide resistance (146 patient-isolates), and 7 SNPs were not previously reported (23 patient-isolates) (*10*–*15*).

There were 34 polymorphisms identified from South Africa, of which 14 (41%, constituting 388 patient-isolates) were present in >1 patient (Table 1). We found that, for 382 (98%) of 388 patients, the RFLP pattern was identical to that of at least 1 other patient with the same *pncA* mutation (Table 1). Moreover, each *pncA* polymorphism was associated with only 1 RFLP pattern in 10 of the 14 polymorphisms. By comparison, 13 RFLP patterns were seen among the 40 patients with a wild-type *pncA* sequence.

Conclusions

In this study, we found that 70% of MDR TB and 96% XDR TB patient-isolates had *pncA* polymorphisms. Given the high likelihood of frameshift mutations resulting in resistance and the high specificity (94%–98%) of *pncA* SNPs for pyrazinamide resistance (*13*,*14*), we estimate that at least 56%–66% of MDR TB and 90%–95% of XDR TB cases from these settings are likely to be resistant to pyrazinamide. Only a small number of mutations were synonymous, previously associated with pyrazinamide susceptibility, or had an SNP for which phenotypic susceptibility has not been previously tested. This finding has implications regarding the effectiveness of empiric use of pyrazinamide for drug-resistant TB or novel treatment regimens. Further studies are needed to fully determine the association of *pncA* mutations with treatment outcomes.

A diversity of *pncA* mutations—69 distinct polymorphisms—were observed among MDR and XDR TB patients, of which none were shared in common between the isolates from South Africa and Georgia. Most *pncA* polymorphisms were unique to individual patients. When the same *pncA* polymorphism was seen in >1 patient, the IS*6110* RFLP pattern was nearly always similar, suggesting that the *pncA* mutation was acquired before transmission. The diversity of polymorphisms underscores previous findings that there is no clear hotspot for *pncA* mutations (*12*), unlike resistance-conferring regions for other TB drugs (e.g., *rpoB*, *katG*) (*11*). Development of rapid molecular tests for pyrazinamide susceptibility may be hampered by the lack of a hotspot for mutations; 1 assay has been developed to detect the full wildtype *pncA* sequence, but its diagnostic accuracy has not yet been adequately tested (*7*).

A limitation of our study is that phenotypic pyrazinamide susceptibility testing was not performed on the sequenced isolates. Nonetheless, correlation with phenotypic testing has been previously reported in the literature for most polymorphisms, enabling us to estimate the proportion likely to be pyrazinamide resistant (*10*–*15*). In addition, the studies that provided these isolates were not specifically designed to be representative of all diagnosed cases of drug-resistant TB; nonetheless, the study populations were carefully selected to provide a high level of generalizability to the broader population. National drug resistance surveys that include pyrazinamide genotypic and phenotypic susceptibility should be designed to confirm these findings.

The high prevalence of *pncA* polymorphisms from geographically disparate countries suggests that guidelines to empirically use pyrazinamide in drug-resistant TB regimens, including shorter MDR TB regimens (*4*), should be reconsidered. Simplified assays to test pyrazinamide susceptibility are needed, although they may be difficult to develop given the genotypic or phenotypic complexities. Considering the potential synergy of pyrazinamide with new TB drugs, routine assessment of pyrazinamide will be increasingly necessary and useful.

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Increase in Tuberculosis Cases among Prisoners, Brazil, 2009–2014[1]

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During 2009–2014, incarceration rates in Brazil rose 34%, and tuberculosis (TB) cases among prisoners rose 28.8%. The proportion of national TB cases that occurred among prisoners increased from 6.2% to 8.4% overall and from 19.3% to 25.6% among men 20–29 years of age.

In 2014, Brazil had the world's fourth largest prison population, with more than half a million prisoners in 1,424 facilities (*1*). Prison conditions include overcrowding, frequent prisoner movement, poor ventilation, and limited access to diagnostic facilities; these conditions favor tuberculosis (TB) transmission (*2*–*5*). Many key factors for progression from TB infection (determined by positive test results) to disease (determined by clinical diagnostic criteria) also are common; these include alcohol and drug abuse, tobacco smoking, undernutrition, and HIV prevalence (*6*). Although surveys have reported high rates of TB among prisoners in Brazil (*6*–*9*), state-level and temporal trends in TB notifications and the contribution of the epidemic in prisons to the overall TB case burden have not been evaluated. To estimate trends in TB cases among prisoners and to identify incarcerated populations at high risk for infection, we analyzed data from the national disease notification database, census data, and administrative data.

The Study

We obtained individual-level data on TB cases reported during 2007–2014 in the Brazil national notification database (Sistema de Informação de Agravos de Notificação; SINAN). A new form for SINAN was introduced in 2007, which led to an increase in documentation of incarceration status from 68% in 2007 to 94% in 2009. Our analysis therefore focuses on data from 2009–2014, when documentation rates were consistently ≥92%. We extracted demographic data on incarceration status, sex, age, and state as well as clinical presentation, HIV status, and sputum smear positivity. This work was approved by the human research protection program at Yale University and the institutional review board at the Federal University of Grande Dourados.

Among prisoners, TB case-patients were defined as persons for whom TB was notified while they were incarcerated, including those in penal institutions awaiting trial or sentencing. To calculate annual TB notification rates (cases per 100,000 persons), we used population estimates for Brazil from the 2010 Census and projections and mid-year incarcerated population data from the Ministry of Justice. To compare TB notification rates between incarcerated and nonincarcerated populations, aggregated at the state level, we used the Pearson product-moment correlation.

During 2009–2014, a total of 38,327 (7.3%) of the 526,569 cases of TB reported in Brazil occurred in prisoners. The total number of cases reported in prisoners increased 28.8%, from 5,556 to 7,157 per year, and the proportion of cases notified among prisoners increased from 6.2% in 2009 to 8.4% in 2014. During the same period, the prison population grew from 409,287 to 579,781, an increase of 41.7%, as overall incarceration rates rose 33.9%, from 214 to 287/100,000 persons. Because of that increase, the rate of TB notifications dropped from 1,357 to 1,234/100,000 for prisoners (9.1% decrease); meanwhile, the rate of TB notifications for nonprisoners dropped 12.2% (from 43.7 to 38.4/100,000 persons). Overall, the mean annual notification rate among prisoners was 31.3 times that of the general population (Table).

HIV status was known for 66% of all TB patients; prevalence was 15.9% among prisoners and 17.4% among nonprisoners (Table). Notification rates varied substantially among states. Average annual 2009–2014 notification rates among prisoners ranged from 225 (Rondônia) to 2,548 (Rio de Janeiro). At the state level, 2014 notification rates were positively correlated (Pearson ρ 0.54; $p = 0.002$) between the nonincarcerated population and prisoners.

Men comprised 93.8% of the prison population and accounted for 91.9% of prison TB cases; 74.7% were 18–39 years of age, compared with only 39.8% in this age range among nonprisoners with TB. In 2014, a total of 25.6% of all men 20–29 years of age were prisoners (state range for

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Table. Annual average data for tuberculosis notifications, by incarceration status and gender, Brazil, 2009–2014

Category	General population			Prisoners			p value, men vs. women*
	All	Men	Women	All	Men	Women	
Population, thousands	194,898	96,546	98,352	490	459	31	<0.0001
Annual no. cases	81,370	53,138	28,227	6,388	5,869	519	<0.0001
Notification rate, cases/100,000 population	42	55	29	1,307	1,281	1,703	0.001
HIV status reported, %†	66.1	66.6	65.3	65.9	65.9	66.0	0.97
HIV co-infection, %‡	17.4	18.2	15.8	15.9	15.2	24.1	0.003
Smear positive, %	55.5	57.2	52.2	65.5	66.4	55.2	<0.0001
Extrapulmonary, %	17.5	16.4	19.5	8.2	7.4	16.7	0.0002
Treatment success, %	66.6	64.4	70.9	69.5	69.8	66.0	0.09

*p values calculated with the Welch *t*-test comparing prisoners' annual data by sex.
†HIV status based on a positive or negative status result or AIDS diagnosis from the time of tuberculosis notification.
‡% HIV co-infection calculated as percentage positive HIV+ results among those with HIV status reported.

all prisoners 0%–47.8%; Figure 1, panels A, B). Although there were far fewer female prisoners, incarceration rates rose faster for women (50.0% vs. 30.2%; Figure 2), and the average annual TB notification rate was higher for female prisoners than for male prisoners (1,703 vs. 1,281; Figure 2). The prevalence of extrapulmonary disease among women was twice that in men (16.7% vs. 7.4%; p = 0.0002). HIV co-infection rates were also higher for female prisoners (24.1% vs. 15.2%; p = 0.003, Table).

Conclusions

Prisoners have been recognized as a group at high risk for TB, but there has been limited assessment of the contribution of recent increases in TB prevalence in the prison population to the overall TB case burden in Brazil. We combined data from a national TB notification database with incarceration data and found that prisons account for a growing proportion of the national TB burden in Brazil. In 2014, a total of 8.4% of all reported cases occurred among prisoners, who represent <0.3% of the population, representing a 35.4% increase in the proportion of TB cases occurring among prisoners in 5 years. Among young men 20–29 years of age, a growing proportion (>25%) of TB cases occurred among those who were incarcerated. For the nonincarcerated population, TB notification rates fell by 12.2% during this period; however, when prisoners were included in assessments, 16.8% of

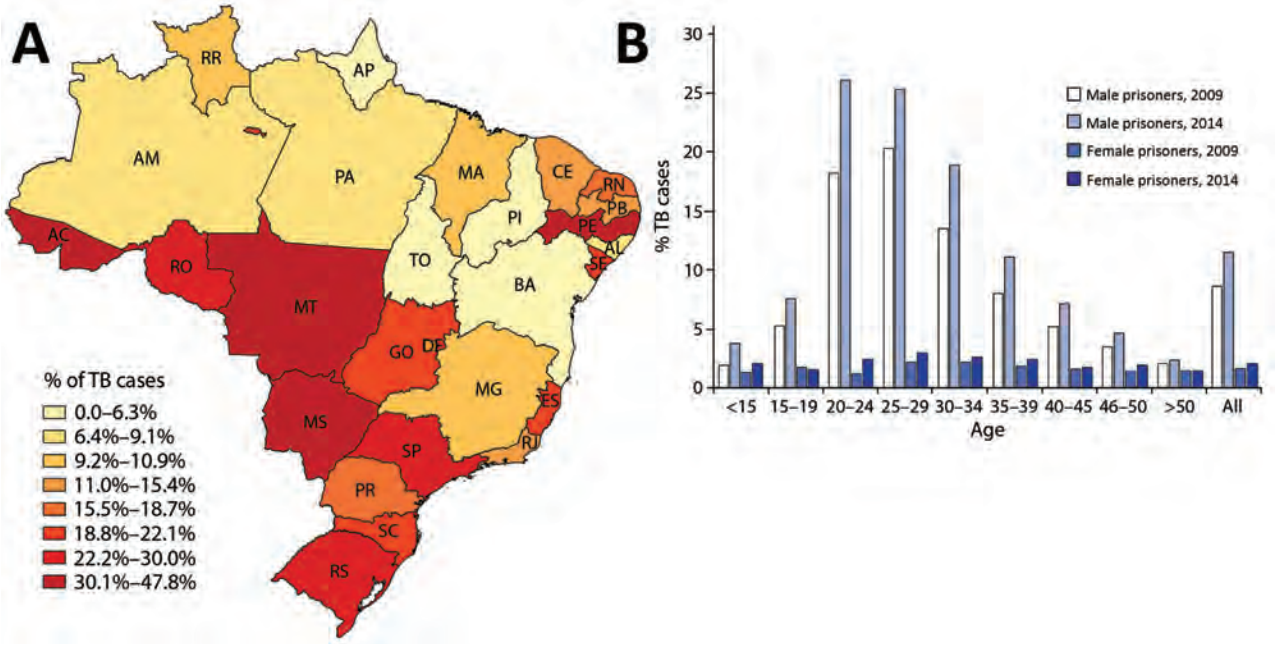

Figure 1. Proportion of tuberculosis (TB) cases among prisoners in Brazil, 2009–2014. A) Geographic distribution by state of the proportions of all TB cases diagnosed among male prisoners ages 20–29. Prisoners comprised 0–47.7% of all TB cases in this age group, with highest rates at the western border of Brazil. B) Sex and age distribution of the proportions of all notified TB cases diagnosed among prisoners in Brazil for 2009 compared with 2014. Prisoners of both sexes represent an increasingly disproportionate percentage of notified cases in all age groups, and male prisoners 20–29 of age represented >25% of cases among the age group in 2014. AC, Acre; AL, Alagoas; AP, Amapá; AM, Amazonas; BA, Bahia; CE, Ceará; DF, Distrito Federal; ES, Espírito Santo; GO, Goiás; MA, Maranhão; MT, Mato Grosso; MS, Mato Grosso do Sul; MG, Minas Gerais; PR, Paraná; PB, Paraíba; PA, Pará; PE, Pernambuco; PI, Piauí; RJ, Rio de Janeiro; RN, Rio Grande do Norte; RS, Rio Grande do Sul; RO, Rondônia; RR, Roraima; SC, Santa Catarina; SE, Sergipe; SP, São Paulo; TO, Tocantins.

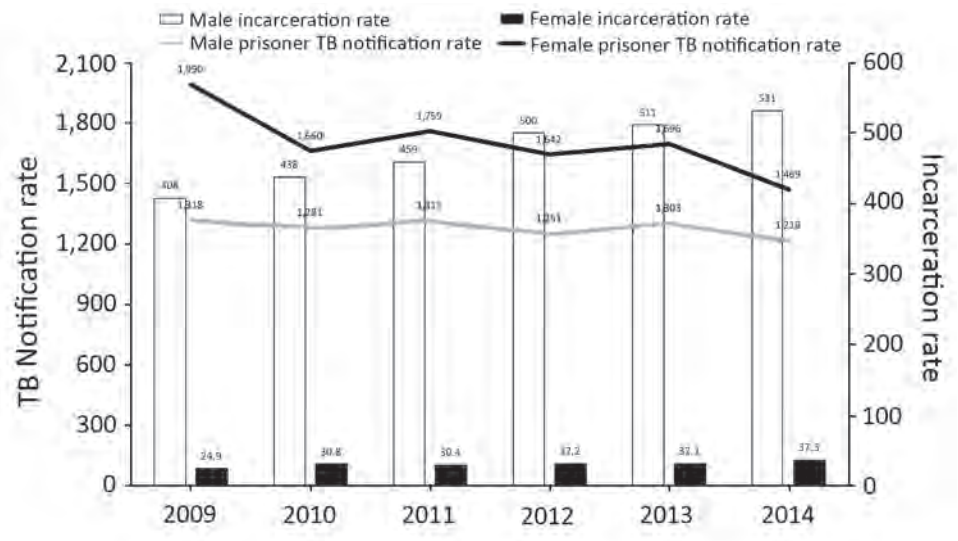

Figure 2. Temporal distribution of incarceration rates (prisoners/100,000 population) and TB notification rates (cases/100,000 prisoners), by sex, Brazil, 2009–2014. There was an appreciable increase in incarceration rates among men (30.2%) and women (50.0%) from 2009 to 2014. Although the incarceration rate of men averaged >15 times that of women, the average TB notification rate for women was higher (1,703 vs. 1,281/100,000 prisoners). TB, tuberculosis

these gains were offset by increasing incarceration rates, as notification rates among prisoners were 31.3 times higher than those for the general population. Failure to address TB in an expanding incarcerated population may be a critical barrier to achieving national targets for TB control.

Our analysis also revealed that female prisoners are at higher risk for TB. Incarceration rates among women rose faster, and TB notification rates and HIV co-infection rates were higher than corresponding rates among men. This finding is in contrast to the overall global epidemiology of TB and to national data for the nonincarcerated population, which demonstrated higher TB notification rates among men than women (50.1 vs. 26.5/100,000 persons; Table). A potential explanation is the higher rate of HIV-associated TB among female prisoners than among men (24.1% vs. 15.2%). We previously reported a higher prevalence of HIV among female prisoners in Brazil (*10*), as have others (*11*,*12*). HIV alone is unlikely to explain the large disparities in TB rates between male and female prisoners; more data are needed to explain the inverted sex disparities (compared with the general population) in TB patients in prisons in Brazil.

This study had several limitations. First, we assessed notification rates at the national and state level; although we observed substantial heterogeneity at the state level, more data on prison characteristics and incarceration rates are needed to understand factors driving TB in prisons. In particular, the relative contribution of individual risk factors for TB reactivation and the effects of prison environments and policies on transmission are poorly understood. Second, our use of data from reported cases may include demographic or diagnostic misclassification. Third, we could not assess cases among recently released prisoners; many prisoners are released without sentencing or after serving sentences of <5 years (*1*), and infections acquired in prison may be diagnosed after prisoners are released. Consequently, the number of cases notified among prisoners (incarcerated at the time of diagnosis) probably underestimates the true effect of prisons on the population-level TB case burden.

Our findings demonstrate that the epidemic of TB in prisons represents a larger challenge to national TB control in Brazil than the threat posed to prisoners' health (*4*,*13*). There is an urgent need to address notification rates in prisons in Brazil by using effective interventions such as active case detection, preventive therapy, and transitional care for released prisoners (*2*,*3*,*5*,*14*). A recent study demonstrated high rates of nonadherence to treatment among prison prisoners in Brazil, underscoring the need to ensure that prisoners complete treatment to prevent transmission within and outside prisons (*15*). In addition to improving access to TB diagnosis and treatment within prisons, addressing rising incarceration rates and improving living condtions that favor transmission in prisons may be critical to combat TB among prisoners (*16*).

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etymologia

Mycobacterium chimaera [mi"ko-bak-tēr'e-əm ki-mēr'ə]

Ronnie Henry

Etruscan bronze statue depicting the legendary monster, the chimera. National Archaeological Museum, Florence. Photograph by Lucarelli (Wikimedia Commons)

Formerly an unnamed *Mycobacterium* sequevar within the *M. avium*–*M. intracellulare*–*M. scrofulaceum* group (MAIS), *M. chimaera* is an emerging opportunistic pathogen that can cause infections of heart valve prostheses, vascular grafts, and disseminated infections after open-heart surgery. Heater–cooler units used to regulate blood temperature during cardiopulmonary bypass have been implicated, although most isolates are respiratory. In 2004, Tortoli et al. proposed the name *M. chimaera* for strains that a reverse hybridization–based line probe assay suggested belonged to MAIS but were different from *M. avium*, *M. intracellulare*, or *M. scrofulaceum*. The new species name comes from the chimera, a mythological being made up of parts of 3 different animals.

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Likely Autochthonous Transmission of *Trypanosoma cruzi* to Humans, South Central Texas, USA

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Chagas disease, caused by *Trypanosoma cruzi*, is a major neglected tropical disease affecting the Americas. The epidemiology of this disease in the United States is incomplete. We report evidence of likely autochthonous vectorborne transmission of *T. cruzi* and health outcomes in *T. cruzi*–seropositive blood donors in south central Texas, USA.

Chagas disease (*Trypanosoma cruzi* infection) is a neglected tropical disease affecting the Americas and a major cause of preventable illness and death, with ≈6–8 million cases worldwide (*1*). This disease can cause progressive cardiac damage postinfection in 30% of infected persons without any initial suggestive clinical symptoms. These latent infections can remain quiescent for decades before manifesting as cardiac complications, including cardiomyopathy, heart failure, and rare cardiac arrest (*2*).

In 2010, US Food and Drug Administration (Silver Springs, MD, USA) issued final guidelines regarding screening of the US blood supply for *T. cruzi* (*3*,*4*). During 2008–2012, screening results showed that 1 in 6,500 donors from an area covering most of the state of Texas were reactive for *T. cruzi* antibodies (*5*). The origin of infection for these donors was unknown. However, high infection rates for reservoir animals and triatomine bug vectors in south central Texas suggested that *T. cruzi* transmission cycles resulting in human infections could occur at a higher frequency than suspected (*6*,*7*). Therefore, we evaluated potential transmission sources and cardiac health of blood donors from south central Texas with *T. cruzi* antibodies.

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The Study

This study was approved by institutional review boards at Baylor College of Medicine (Houston, TX, USA), the Gulf Coast Regional Blood Center (Houston), and the South Texas Tissue and Blood Center (San Antonio, TX, USA). Blood donors residing in the greater San Antonio, Texas, area who had *T. cruzi* antibodies detected by PRISM Chemiluminescent Immunoassay (Abbott Laboratories, Chicago, IL, USA) or Ortho T. cruzi ELISA (Ortho-Clinical Diagnostics Inc., Raritan, NJ, USA), and a positive result for a Radioimmune Precipitation Assay (Quest Diagnostic Laboratories, Madison, NJ, USA) or an ESA Chagas Test (Abbott Laboratories) during January 1, 2008–December 31, 2014, were invited to participate in the study. Persons previously enrolled in a Houston-based *T. cruzi*–seropositive blood donor project were not eligible for this study (*8*).

Letters in English and Spanish were sent to donors who had *T. cruzi* antibodies by the blood centers for this study. Those who agreed to participate provided informed consent. We performed 3 procedures: 1) blood collection for additional serologic screening, 2) structured interview to assess potential transmission sources and health, and 3) 12-lead resting electrocardiogram (ECG) (*8*).

Blood specimens were used for serologic testing (Table 1). We defined a case of *T. cruzi* infection if donor screening test results and ≥2 serologic test results were positive. Likely autochthonous *T. cruzi* infection was defined in a case-patient who had no major travel to a Latin American country (lasting >2 weeks or that included an overnight stay in a rural region), not having been born in Latin America, and not having a mother born in Latin America (*4*,*8*,*10*). Congenital transmission from a maternal grandmother (2 contiguous congenital infections) cannot be ruled out with this case definition but is unlikely given the low risk for congenital transmission (*7*). Occupations, residential history, and clinical health information were reviewed in a questionnaire. ECG readings were interpreted by a board-certified cardiologist.

For persons who donated blood in the greater San Antonio area during the study period we found that 61/256,801 donors had positive serologic results for *T. cruzi* infection (1/4,200 donors had positive serologic results for *T. cruzi* infection by 2 assays). Seventeen (28%) of these donors were

Table 1. Characteristics for 14 case-patients infected with *Trypanosoma cruzi*, south central Texas*

Donor no./age, y/sex	Likely autochthonous transmission†	Blood bank serologic test results‡		Study serologic test results§					ECG results¶	Concurent condition
		PRISM or ORTHO	RIPA or ESA	Hemagen	Stat-Pak	DPP	EIA	TESA		
1/83/M	Yes	+	+	+	+	+	+	+	Primary AV block, atypical incomplete right BBB, lateral asymmetric T inversion	None
2/61/F	Yes	+	+	+	+	+	+	+	Inferolateral asymmetric T inversion	Hypertension
3/71/M	Yes	+	+	+	+	+	+	+	LAD, nonspecific ST/T wave abnormality	Kidney failure, hypertension
5/19/M	Yes	+	+	+	+	+	+	+	Normal	None
6/60/M	Yes	+	+	+	+	+	+	+	Primary AV block	Diabetes, hypertension
7/56/F	Yes	+	+	+	+	+	–	+	Minimum voltage criteria for LVH	None
8/52/M	Yes	+	+	+	+	+	+	+	LAD	Parkinson's disease
9/25/F	Yes	+	+	+	+	+	+	+	Normal	None
10/51/F	Yes	+	+	+	+	+	Ind	+	Normal	Heart attack
11/52/F	Yes	+	+	+	+	+	+	+	Normal	None
12/45/M	No	+	+	+	+	+	+	+	Normal	Borderline diabetes
13/35/F	No	+	+	+	+	+	+	+	Normal	None
14/34/F	No	+	+	Ind	+	+	+	+	Nonspecific T wave change	None

*Demographic information, likely autochthonous transmission, and concurrent conditions were determined through case-patient interview. ECG, electrocardiogam; Ind, indeterminate; +, positive; –, negative. Test results were based on manufacturers' protocols for serologic testing.
†Donors listed as showing autochthonous transmission (donors 12–14) reported living in Mexico or Chile.
‡ESA, Chagas Test (Abbott Laboratories, Chicago, IL, USA); ORTHO, *T. cruzi* ELISA (Ortho-Clinical Diagnostics Inc., Raritan, NJ, USA); PRISM, Chemiluminescent Immunoassay (Abbott Laboratories); RIPA, radioimmune precipitation assay (Quest Diagnostic Laboratories, Madison, NJ, USA).
§DPP, dual path platform immunochromatographic confirmation assay (Chembio, Medford, NY, USA); EIA, Chagatest recombinant v3.0 enzyme inmmunoassay (Wiener, Rosario, Argentina); Hemagen; Chagas EIA Kit (Hemagen Diagnostics, Inc., Columbia, MD, USA); Stat-Pak, Chagas immunochromatographic assay (Chembio, Medford, NY, USA); TESA, trypomastigote excreted or secreted antigen immunoblot. Hemagen, Stat-Pak, and DPP were performed at Baylor College of Medicine, (Houston, TX, USA), and EIA and TESA were performed at the Centers for Disease Control and Prevention (Atlanta, GA, USA).
¶Results were determined from readout of a resting 12-lead ECG and interpreted by a board-certified cardiologist. AV, atrioventricular; LAD, left axis deviation; LVH, left ventricular hypertrophy; RBBB, right bundle branch block.

enrolled in the study; additional serologic testing confirmed that 14 had antibodies against *T. cruzi* when the study began (Table 1). These persons had a mean age of 47 years (range 19–83 years); 50% were Hispanic, 50% were non-Hispanic white, and 50% were men. For 3 persons whose blood donor testing results were not confirmed by further serologic testing, 2 were non-Hispanic and 1 was Hispanic (2 women and 1 man); mean age was 51 years. Because of the blinded nature of study recruitment, we cannot identify demographic data for persons who received the letter and chose not to participate.

Likely autochthonous transmission of *T. cruzi* was suspected for 11 (79%) of 14 persons, as defined by study criteria. These 11 persons had a mean age of 50 years; 7 were non-Hispanic whites, and 6 were men. Remaining data presented will concern only the 11 newly identified persons with likely autochthonous infections.

A structured interview adapted from a questionnaire used by the Centers for Disease Control and Prevention (Atlanta, GA, USA), the American Red Cross (Washington, DC, USA), and Blood Systems, Inc. (Scottsdale, AZ, USA) was used to identify risk factors for *T. cruzi* infection (*4*). Because of the lifelong nature of infection and antibody-based diagnostics used, a specific time of infection could not be established for each case-patient. However, we identified common themes for transmission risks for this cohort. Most (91%) case-patients with likely autochthonous infection reported a history of living in a rural community (Figure). Residence in rural communities could pose a risk for *T. cruzi* transmission because this setting might lead to close proximity with sylvatic transmission cycles involving the vector and infected animals (*11*).

Although recreational activities or occupations associated with outdoor exposure were reported among our cohort, we obtained evidence suggesting that opportunities for transmission might be occurring near homes in rural communities (Table 2). Specifically, patients with likely autochthonous infections reported seeing the vector around their current or previous residence (36%), and had animal housing near their homes (73%). An extensive history of outdoor recreational activities of hunting and

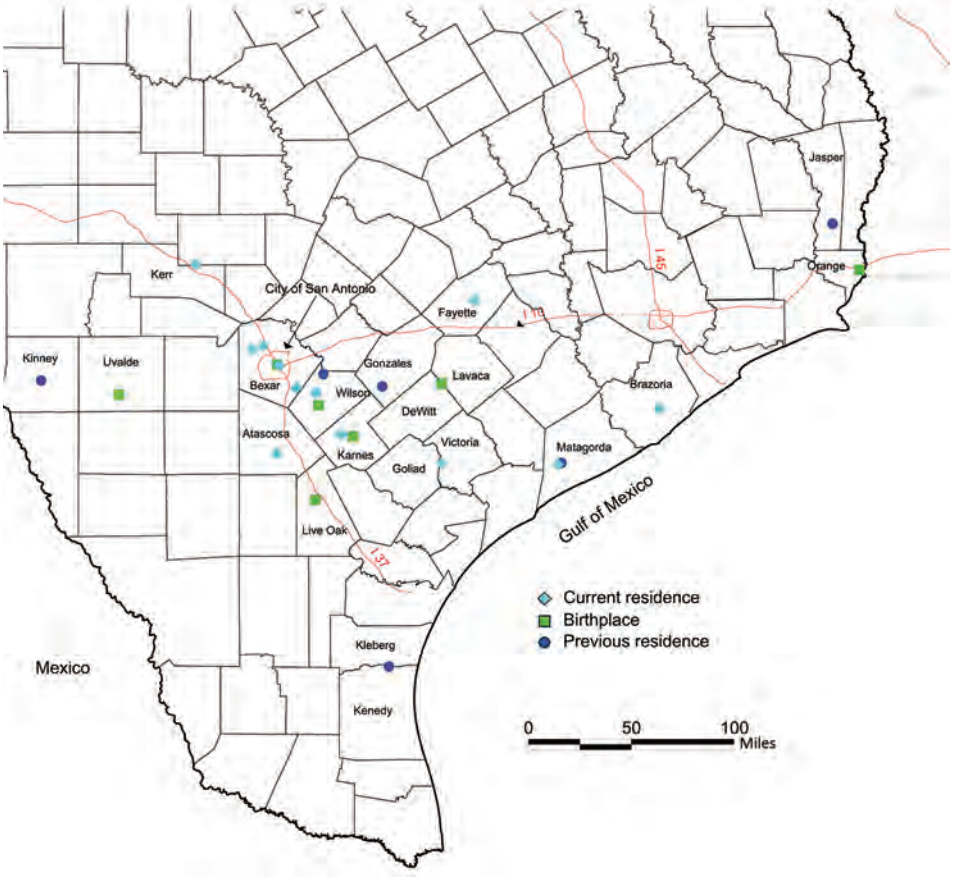

Figure. Current and previous residences of case-patients with likely autochthonous infection with of *Trypanosoma cruzi*, south central Texas, USA, including 11 autochthonous donors with current residence and birthplace. County boundaries are shown. Previous residences in Texas were chosen if the case-patient reported living in the location ≥5 years.

camping, which has been suggested as a high-risk activity for *T. cruzi* transmission in the southern United States, was less common then expected (36%) (*8*,*12*). Two of 11 case-patients reported agricultural jobs and staying in substandard housing during the harvest season, thereby introducing the potential for disease transmission from triatomines in the home.

Five case-patients reported a lack of knowledge of Chagas disease by their primary care physicians. Some case-patients were provided with misinformation, reporting having been told that their screening test result must be false positive because they had no travel history. Furthermore, only 2 case-patients were offered treatment before enrollment in the study. One case-patient reported that, despite seeking treatment for >1 year, he was unable to find a physician able and willing to help.

This finding is particularly problematic given that a large proportion (6 of 11) of this cohort had abnormal ECG readings possibly attributable to Chagasic cardiac disease. Although precise cardiac etiologies could not be determined, prevalence of ECG abnormalities was higher than that for population-based studies (*13*,*14*); common findings included atrioventricular block and left axis deviation (Table 1). A previous report also highlighted the same lack of physician awareness of Chagas disease in Texas, despite patients having positive serologic screening results and cardiac manifestations (*8*).

Conclusions

Given the low level of participation of seropositive blood donors, results of this study are limited to persons who participated and might not represent the larger Texas blood donor population or general population. Also, because a 7-year span separated initial screening and enrollment in this study, it is difficult to identify why 3 persons who were initially positive by blood bank screening had discordant results during the study. At follow-up, participating persons were tested with available Centers for Disease Control and Prevention assays, Food and Drug Administration–approved screening, or supplemental tests.

Our study adds 11 cases of likely domestically acquired *T. cruzi* infection to the increasing body of evidence for autochthonous Chagas disease transmission in the southern United States. Combined with previous studies indicating a high rate of *T. cruzi* infection in triatomine vectors and mammalian reservoirs in this area, our study shows that south central Texas could be a focal point for

Table 2. High-risk activity profile for 11 case-patients with likely autochthonous infection of *Trypanosoma cruzi*, south central Texas, USA*

Case-patient	Birthplace/former residence	Current residence	Occupational	Recreational camping	Recreational hunting
1	+++	+	++	++	+
2	+++	0	0	+	0
3	+	+	+++	+	+
4	0	0	0	++	0
5	+++	0	+	+	0
6	+++	+++	+	0	++
7	+	+++	+++	+	0
8	++	0	++	0	++
9	0	+++	+	0	0
10	+	+	0	0	0
11	++	+++	0	+	0

*Risk was determined through administration of a patient survey. No risk (0) was defined as not living in a rural area and having no history of outdoor occupation or recreational activities. Low risk (+) was defined as ever living in a rural area, having an outdoor occupation, or engaging in hunting or camping in an area with known triatomine activity. Moderate risk (++) was defined as, in addition to low-risk activities, an extensive history of these activities (>1 y), or having slept in a tent in a rural part of Texas. High risk (+++) was defined as, in addition to moderate-risk activities, reporting 1 of the following: reported seeing triatomines, had collective animal housing around the property, or lived or slept in substandard housing.

endemic disease transmission (*7*,*15*). We also identified a major knowledge gap for Chagas disease, which highlights the need for enhanced public health campaigns targeting clinicians and the general population in south central Texas.

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Dr. Gunter is a postdoctoral fellow at Baylor College of Medicine, Houston, TX. Her research interests include the epidemiology of Chagas disease in the United States and host–parasite interaction.

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Mycobacterium tuberculosis in Wild Asian Elephants, Southern India

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We tested wild Asian elephants (*Elephas maximus*) in southern India and confirmed infection in 3 animals with *Mycobacterium tuberculosis,* an obligate human pathogen, by PCR and genetic sequencing. Our results indicate that tuberculosis may be spilling over from humans (reverse zoonosis) and emerging in wild elephants.

Infection with *Mycobacterium tuberculosis* in domestic and wild animals of various species living in close contact with humans has been reported (*1*). Elephants in captivity are known to be susceptible to infection with *M. tuberculosis*, and there is a potential for transmission of *M. tuberculosis* between humans and elephants (*2–4*). In 2013, a case of tuberculosis (TB) in a wild elephant in Africa, which had been under human care, was reported (*5*), after which another case in a wild Asian elephant in Sri Lanka was reported (*6*). Habitat encroachment and competition for resources brings wild elephants into closer contact with humans, providing opportunities for zoonoses and reverse zoonoses to occur and for a previously unknown pathogen to emerge in captive free-ranging and wild elephant populations.

The Study

In March 2007, an emaciated wild bull elephant, estimated to be 20 years of age, died shortly after it was found recumbent in the Muthanga range of the Wayanad Wildlife Sanctuary in southern India (case 1). Postmortem examination revealed purulent exudates throughout the lungs, an enlarged liver, enlarged mesenteric lymph nodes, and surface nodules containing caseated yellowish-white material (Figure 1). We found serosanguinous fluid in the pericardial sac and slightly hypertrophied heart ventricles. We saw focal areas of necrosis in the renal cortices but noted no other gross lesions. Ziehl-Neelsen staining of lung, liver, kidney, and mesenteric lymph node impression smears revealed numerous acid-fast bacilli. We confirmed the presence of *M. tuberculosis* by using PCR amplification of the targeted bacterial genome, gel documentation of the amplified products, and sequencing.

Subsequently, a surveillance program was initiated (until March 2014), and all fresh elephant carcasses in the study area were examined for evidence of TB (n = 88). In May 2010, a bull elephant, ≈30 years of age, was found dead in the Kurichiyat range (case 2). Postmortem examination revealed extensive caseated lesions in the lungs (Figure 2) and mild mesenteric lymph node hypertrophy. In May 2013, TB infection was diagnosed in a bull ≈40 years of age that was found in the same forest range and had extensive caseated lung lesions (case 3). Both bulls were emaciated.

We fixed samples for histopathological studies in 10% formol saline and embedded them in paraffin. We found numerous acid-fast organisms in lung impression smears and tissue sections. Granulomatous lesions encapsulated by connective tissue with aggregated macrophages and central areas of necrosis were seen during histopathologic examination of the lungs for all 3 cases and of the kidney and liver in case 1. Langerhans-type giant cells were observed in cases 2 and 3 but not in case 1.

Tissues for molecular studies were collected in absolute alcohol. We extracted total DNA from tissues by using DNeasy Blood & Tissue Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's protocol. DNA was subjected to a tetraplex PCR to differentiate between *M. tuberculosis* complex and nontuberculous mycobacteria. DNA was subjected to amplification and sequencing of the 3 target regions separately, 16S–23S internal transcribed spacer region, hsp65, and rpoB separately (*7*). *M. tuberculosis* H37Rv and *M. bovis* bacilli Calmette-Guérin genomic DNA was used as control DNA for the PCR studies.

We observed the expected 4-band pattern after tetraplex PCR. As the MTP40 fragment was amplified, *M. bovis* was ruled out because the *plcA* gene (mtp40), one of the members of the *plc* family of genes that code for the phospholipase C enzyme, is deleted in the *M. bovis* and *M. bovis* bacilli Calmette-Guérin RD5 region (*8*). Sequences that were generated were assembled and

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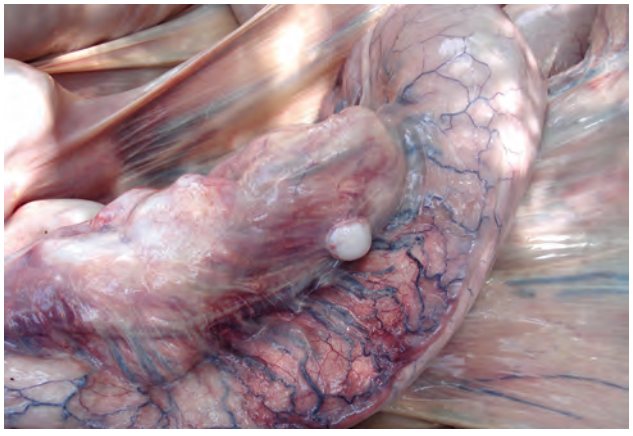

Figure 1. Intestine from a wild bull elephant, estimated at 20 years of age, Wayanad Wildlife Sanctuary, India, 2007. Multiple white-to-tan discrete nodules (granulomas) are protruding from the serosal surface, and less well-defined areas of pale discoloration are visible within the intestinal wall. Serosal blood vessels are markedly dilated, tortuous, and congested

edited by using the alignment software Seqscape (http://www.seqscape.software.informer.com). BLAST (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi) analysis of the edited sequences revealed that the elephant sequences showed 100% similarity with the *M. tuberculosis* genome fragment. We also used DNA for large sequence polymorphism analysis to determine the lineage of *M. tuberculosis* using RD239 and RD750 primers (*9*,*10*). The genomic deletion analysis revealed a deletion in RD239, which is characteristic of the Indo-Oceanic lineage (*10*), also referred to as the East African–Indian lineage (*11*).

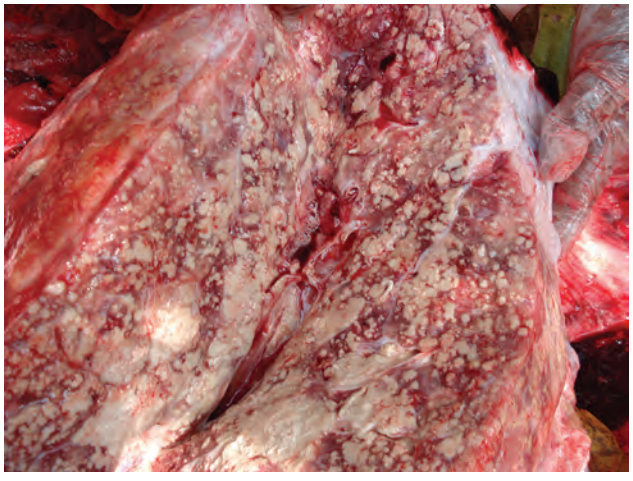

Figure 2. Lung from a bull elephant, estimated at 30 years of age, Kurichiyat Range, India, 2010. Note the multifocal to coalescing pale tan-to-white firm nodules (granulomas) effacing much of the lung parenchyma. Some areas of white chalky mineralization are also present.

Conclusions

There are reports of mycobacterial infections in captive elephants in India from as early as 1925 (*12*). We report *M. tuberculosis* infection in wild elephants in India. In this study, 3 (3.4%) of 88 elephants undergoing postmortem examination were confirmed to be infected with *M. tuberculosis*. All 3 animals were emaciated, and we considered TB to be the cause of death.

The close interaction between humans and captive elephants is presumed to be a key risk factor for the interspecies transmission of TB. The epidemiology of TB among wild elephants, now documented in 3 countries, has yet to be elucidated. In our study, there were no known captive elephant releases or reintroductions into the study area, and the interaction between captive and wild elephants is considered negligible. However, native tribes do live within the park; many tribal members are employed by the forest department for protection and ecotourism activities. Tourists may visit specified areas only under supervision; there are no overnight facilities. Human–elephant conflict is a problem; most conflicts are caused by resident bulls. All 3 TB cases reported here were in bulls. Exposure of bulls to humans infected with TB during conflict activities is a possible explanation.

More than 3,000 native cattle reside within the sanctuary, cared for by the Animal Husbandry Department, Kerala State. No cases of TB among cattle have been reported. Cattle would be more likely to be infected with *M. bovis* than with *M. tuberculosis*, but comprehensive testing would be informative. Cattle living in close proximity to TB-infected humans can become infected with *M. tuberculosis* (*13*). Whether such infected cattle could then transmit *M. tuberculosis* to elephants through contamination of shared grazing lands is yet another research question.

The *M. tuberculosis* complex is thought to have emerged as a human pathogen in Africa rather than arising from an animal source (*14*). Although the epidemiology has not been defined, our study and previous reports indicate that *M. tuberculosis* appears to be spilling over into elephants (reverse zoonosis) and emerging among wild elephant populations. Although these cases may have resulted from individual introductions, if *M. tuberculosis* becomes established, wild elephants and other susceptible species will be at risk.

Ecologic, environmental, or demographic factors that place animals or humans at increased contact can contribute to disease emergence. Certainly, the increased human–elephant conflict in India and other Asian elephant range countries attests to the narrowing interface between humans and elephants. This study suggests that *M. tuberculosis* is emerging in the largest single population of Asian elephants in India. Continued surveillance in India and other Asian elephant range countries is warranted.

Acknowledgments

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Mycobacterium chimaera in Heater–Cooler Units in Denmark Related to Isolates from the United States and United Kingdom

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Mycobacterium chimaera was present at high rates (≥80%) in heater–cooler units (HCUs) from all 5 thoracic surgery departments in Denmark. Isolates were clonal to HCU-associated isolates from the United States (including some from patients) and United Kingdom. However, *M. chimaera* from 2 brands of HCU were genetically distinct.

Based on reports from 2015 (*1*,*2*), the European Centre for Disease Prevention and Control issued a Rapid Risk Assessment alert on April 30, 2015, associating invasive cardiovascular infections with *Mycobacterium chimaera* in water tanks of heater–cooler units (HCUs) used during open-chest heart and vascular surgery (*3*). Subsequently, additional cases from Europe (*4*) and the United States potentially associated with HCUs have been described (*5*–*7*). Preliminary data indicate that the isolates from the patients, the HCUs in hospitals, and the HCUs at the manufacturer are similar (*8*). The aim of this study was to determine *M. chimaera* prevalence in Denmark HCUs and, if present, phylogenetically characterize and quantify the strains.

The Study

Statens Serum Institut, the Danish Patient Safety Authority, and the Danish Medicines Agency decided to investigate all the HCUs in Denmark. Approval from a human or animal research ethics board was not required to conduct this study. The infection prevention control units and thoracic surgery department staff from 5 local hospitals were instructed to collect water and biofilm samples from the HCUs and send them to Statens Serum Institut for testing. In brief, a water culture method adapted for low concentrations of mycobacteria with high concentrations of contaminants was used, and mycobacterial isolates were identified by internal transcribed spacer sequencing (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/23/3/16-1941-Techapp1.pdf). One *M. chimaera* isolate from each thoracic surgery department and one unrelated patient isolate were subjected to whole-genome sequencing (WGS). A total of 10 million paired-end Illumina sequencing reads (Illumina Denmark ApS, Copenhagen, Denmark) were deposited in the European Nucleotide Archive under study number PRJEB18427.

M. chimaera was found in 18/21 (86%) HCUs, representing all 5 thoracic surgery departments in Denmark (Table). Four sites used the Sorin 3T HCU (Sorin Group, Arvada, CO, USA); 14/16 (88%) units contained *M. chimaera*. One site used Maquet brand HCUs (Maquet, Wayne, NJ, USA), 4/5 (80%) units contained *M. chimaera*. The strain *M. gordonae* was found irregularly throughout the HCUs (Table). Both water and biofilm samples could be cultured and were equally effective for the detection of mycobacteria. We used the filter culture method for quantitative analysis purposes and for a simpler workflow. However, the quantitative culture results were poor quality because the analytic sensitivity was low and many samples were heavily contaminated (Table; online Technical Appendix Figure).

WGS analysis (Figure) showed that the 4 isolates from the Denmark Sorin 3T HCUs were nearly identical (<3 single nucleotide polymorphisms [SNPs]). Conversely, the isolate collected from the Maquet HCU was genetically distinct, showing 47–49 SNP differences compared with the isolates from the Sorin 3T HCUs. The unrelated patient isolate was not closely related to the HCU isolates (30–37 SNP differences).

We compared the sequences we collected with 31 other sequenced *M. chimeaera* isolates previously collected (2009–2016) and available in the European Nucleotide Archive (http://www.ebi.ac.uk/ena). This dataset comprised 3 non–HCU-associated patient isolates from Ireland; 9 Sorin 3T HCU isolates and 11 HCU-associated patient isolates from Pennsylvania and Iowa, USA (*6*); and 8 HCU water sample isolates from the United Kingdom (accession nos. PRJNA294775, PRJNA344472, PRJNA345021, and PRJNA324238 for the 4 groups, respectively). Unexpectedly, the *M. chimaera* sequences from the Denmark Sorin 3T HCUs were nearly identical to the isolates from the United States and United Kingdom (median difference 3 SNPs; interquartile range [IQR] 1–5 SNPs) and were similar to all Sorin 3T-associated patient isolates (median difference 6 SNPs; IQR 3–9 SNPs). We saw a distinctly closer relationship between the isolates from Denmark Sorin 3T HCUs and the isolates from UK and US HCUs than between Denmark Sorin 3T

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Table. Identification of *Mycobacterium* spp. from water and biofilm samples taken from heater–cooler units from 5 heart surgery centers, Denmark, July–October 2015*

Heater–cooler unit	Water sample results	Biofilm sample results	Quantitative culture, CFU/L†
A1	*M. chimaera*	NA	100
A2	*M. chimaera*	NA	Mold
A3	*M. chimaera*	NA	60
B1	*M. chimaera*	NA	Mold
C1	*M. chimaera*	*M. chimaera*	0
C2	*M. gordonae*	*M. gordonae*	9
C3	*M. chimaera*	*M. chimaera*	Mold
C4	*M. gordonae*	*M. gordonae*	57
D1	*M. chimaera*	*M. chimaera*	>1,000
D2	*M. chimaera*	NA	Mold
D3	*M. chimaera*	*M. chimaera*	Mold
D4	*M. chimaera*	*M. chimaera*	>1,000
D5	*M. chimaera*	*M. chimaera*	Mold
D6	*M. chimaera*	NA	Mold
D7	*M. chimaera*	*M. chimaera*	Mold
D8	*M. chimaera*	*M. chimaera*	Mold
E1	*M. gordonae*	*M. chimaera*	0
E2	*M. chimaera*	*M. chimaera*, *M. gordonae*	0
E3	*M. chimaera*	*M. chimaera*	0
E4	*M. chimaera*	*M. chimaera*, *M. gordonae*	300
E5	No growth	NA	0

*NA, not available.
†Bacterial concentration of the original sample.

HCUs and the unrelated Denmark or Ireland patient isolates or the Denmark Maquet HCU isolate (Figure).

Overall, the 32 isolates associated with the Sorin 3T HCUs (online Technical Appendix Table) were found to have 15 common SNPs and 0–18 SNP differences between any 2 isolates (median difference 5 SNPs; IQR 3–8 SNPs). These findings support the conclusion by Haller et al. that *M. chimaera* from the Sorin 3T HCUs have a common source (*8*). The *M. chimaera* sequences from the UK HCU water samples were genetically nearly identical to the US and Denmark isolates; we therefore conclude that the UK isolates also originated from Sorin 3T HCUs.

No patients with *M. chimaera* infections associated with open-chest surgery have been suspected or detected

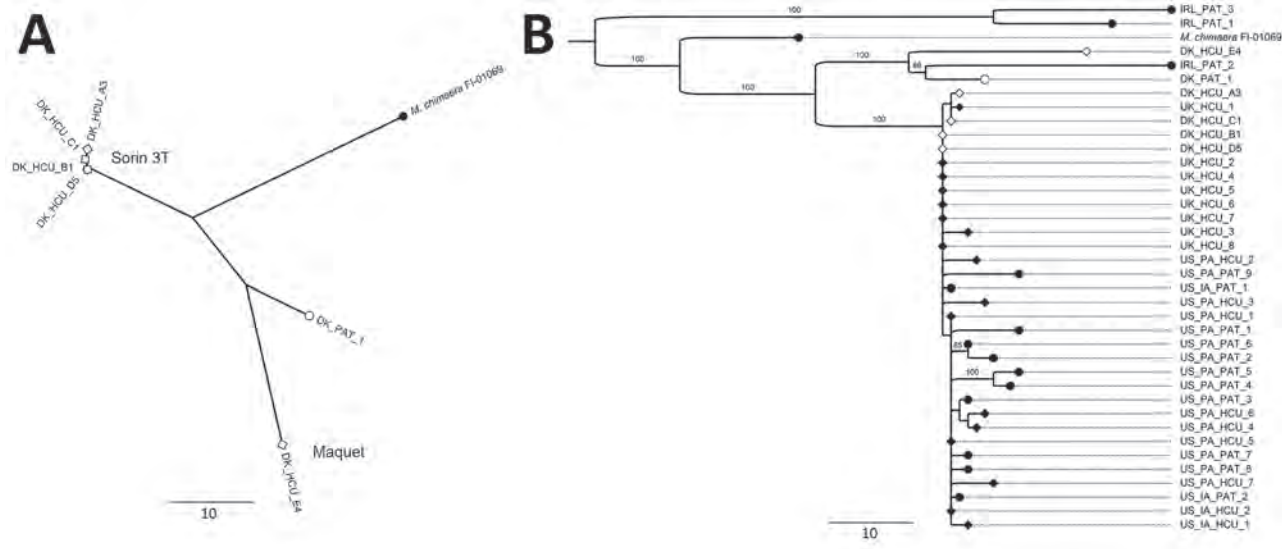

Figure. Maximum parsimony phylogenies showing the relationship between *Mycobacterium chimaera* isolates found in patients (circles) and heater–cooler units (HCUs; diamonds) in Denmark. *M. chimaera* strain FI-0169 (accession no. PRJNA356276) was included for reference. A) Tree showing isolates from Sorin 3T (Sorin Group, Arvada, CO, USA) and Maquet (Maquet, Wayne, NJ, USA) HCUs and a non–HCU-associated isolate from a patient (PAT) in Denmark (DK). B) Phylogenetic tree comparing isolates from Denmark to 31 isolates collected in 3 other countries (Ireland [IRL], United Kingdom, and United States) and retrieved from the European Nucleotide Archive (http://www.ebi.ac.uk/ena). Isolates from Denmark are indicated by open symbols and isolates from other countries by solid symbols. Branch values indicate percentwise bootstrap support (only >70% support is shown), based on 100 replicates. IA, Iowa; PA, Pennsylvania. Scale bars represent a difference of 10 single-nucleotide polymorphisms.

in Denmark. Searching the International Reference Laboratory of Mycobacteriology database, which includes all mycobacteria cultures in Denmark, from 1991 to 2016, we found no records of *M. avium* complex isolates from patients that had an open-chest operation.

Following our findings, 1 thoracic surgery department decided to keep the HCUs in the operating theater but encased them in housings with separate ventilation. Two departments were unable to take the HCUs out of the theaters but decided to move the HCUs as distant as possible from the patients and decontaminate more frequently. Two of the departments had their HCUs outside the operating room already and therefore kept their policies regarding HCUs.

Conclusions

We found that *M. chimaera* was present in most HCUs in Denmark. Isolates from Sorin 3T brand HCUs were identical to the HCU isolates from the United States and the United Kingdom, and thus they appear to have the same origin. Because all 5 of the thoracic surgery departments in Denmark had contaminated HCUs and because mycobacterial contamination has been reported in multiple published studies during 2015–2016 (*4*–*6*), we find it likely that most Sorin 3T HCUs made in the past 8–10 years potentially are contaminated by the same *M. chimaera* strain. In addition, because 80% of the Maquet HCUs also contained *M. chimaera*, although phylogenetically different from the Sorin 3T strains, we suggest mycobacterial contamination might be a general problem for HCUs.

Dr. Svensson specializes in clinical microbiology and works at Statens Serum Institut in Denmark as Technical Director for Diagnostics at the International Reference Laboratory of Mycobacteriology. His research interests are molecular epidemiology, mycobacteria, and pharmacodynamics of antibiotics.

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Rhodococcus Infection in Solid Organ and Hematopoietic Stem Cell Transplant Recipients[1]

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We conducted a case–control study of 18 US transplant recipients with *Rhodococcus* infection and 36 matched controls. The predominant types of infection were pneumonia and bacteremia. Diabetes mellitus and recent opportunistic infection were independently associated with disease. Outcomes were generally favorable except for 1 relapse and 1 death.

Rhodococcus, a gram-positive coccobacillus, has been isolated from water, soil, and the manure of herbivores. It is a facultative intracellular pathogen that survives in host macrophages. Immunosuppressive medications that compromise cell-mediated immunity can predispose to infection (*1*,*2*).

Our knowledge of disease characteristics among transplant recipients is limited to case reports (*3*–*8*). With the increase in organ transplantation and improved survival of transplant recipients, the incidence of disease will likely increase in the coming years. In this study, we sought to describe characteristics, risk factors, and outcomes of *Rhodococcus* infection among solid organ transplant (SOT) and hematopoietic stem cell transplant (HSCT) recipients in the United States.

The Study

We conducted a case–control study at 8 US medical centers during January 2000–December 2012. The study was approved by appropriate Institutional Review Boards. Case-patients were those with clinical or radiographic features of infection and positive culture results for *Rhodococcus* spp. Identification of the organism was performed by using biochemical methods at microbiology laboratories of participating institutions. At the discretion of laboratory staff, identification was confirmed by using 16S rRNA sequencing.

Controls received a similar organ within 3 months before or after the index case-patient at the same center and did not show *Rhodococcus* infection. Each case-patient was matched with 2 controls. Allogeneic and autologous HSCT recipients were matched to recipients of the same type.

We used descriptive statistics to summarize results for the cohort. Conditional logistic regression was used to evaluate risk factors for infection. Factors associated with disease less than the 0.10 significance level for univariate analysis were included in the multivariate model. Statistical software Stata/SE version 13.1 (StataCorp LP, College Station, TX, USA) was used.

We identified 18 patients with *Rhodococcus* spp. infection (Table 1, https://wwwnc.cdc.gov/EID/article/23/3/16-0633-T1.htm). Mean age was 55 (range 3–78) years. Six patients underwent HSCT (5 allogeneic, 1 autologous) and 12 SOT (4 heart, 4 lung, 3 kidney, 1 liver). Median time from transplant to infection was 5 (range 2–54) for HSCT recipients and 28 (range 3–237) months for SOT recipients. Infection occurred within the first year posttransplant for half of the patients. Five (39%) of 13 patients were living on a farm or had known contact with horses; exposure history was unknown for 5 patients.

Median time to diagnosis after onset of symptoms was 20 (range 2–67) days. This median was determined mainly by the time that the patient sought medical attention and the time of clinical specimen collection. At the time of diagnosis, 3 (17%) patients were managed in outpatient settings, 12 (67%) in inpatient wards, and 3 (17%) in intensive care units.

The predominant infections were pneumonia (61%, 11/18) and bacteremia (56%, 10/18). Bacteremia was secondary to pneumonia for 4 patients and catheter-associated for 4 patients. Fever occurred in half of the patients. Patients with pneumonia had dyspnea (45%, 5/11), cough (70%, 7/10), sputum production (20%, 2/10), and chest

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[1]Results from this study were presented in part at IDWeek 2012, October 17–21, 2012, San Diego, California, USA.

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pain (30%, 3/10). None had hemoptysis. Lung disease was infiltrative in 8/11 (73%) patients, nodular in 8/11 (73%), and cavitary in 2/11 (18%). Median neutrophil count at diagnosis was 4,133/mm^3 (range 532–16,468/mm^3), and median lymphocyte count was 705/mm^3 (range 90–3,350/mm^3). Species identification was performed for 9 isolates (8 *R. equi* and 1 *R. corynebacterioides*). We found no significant difference in incidence of preceding opportunistic infection between SOT and HSCT recipients (33% vs. 50%; p = 0.62).

Infected patients were matched with 36 controls. Univariate analysis showed that type of immunosuppression, augmented immunosuppression, increased levels of tacrolimus or cyclosporine, and trimethoprim/sulfamethoxazole (TMP/SMX) prophylaxis were not associated with infection. Multivariate analysis showed that diabetes mellitus (p = 0.041) and recent opportunistic infection (p = 0.045) were independently associated with infection (Table 2).

All isolates tested were susceptible to vancomycin (5/5), rifampin (5/5), linezolid (9/9), and imipenem (7/7). Fourteen percent (1/7) were susceptible to amoxicillin/clavulanate; 29% (2/7) to ceftriaxone; 55% (6/11) to TMP/SMX; 70% (7/10) to tetracycline or minocycline; 75% (6/8) to azithromycin or clarithromycin; and 80% (4/5) to levofloxacin, moxifloxacin, or gatifloxacin. Four isolates were resistant to penicillin and 1 was resistant to clindamycin.

Most patients received combination treatment with 2–3 antimicrobial drugs (Table 1). Most commonly used drugs in the initial regimen were vancomycin, a fluoroquinolone, or a carbapenem. Median duration of treatment was 1 month (range 2 weeks–7 months) for patients with cathether-associated bacteremia and 6 months (range 2–60 months) for patients with all other infections. Immunosuppression was decreased in 44% (7/16). The patient with a pacemaker pocket infection had the device removed.

Median follow-up period was 17 (range 1–84) months. One allogeneic HSCT recipient with *R. equi* pneumonia died of respiratory failure 13 days after diagnosis. He was receiving effective treatment with levofloxacin and TMP/SMX. One allogeneic HSCT recipient with bacteremic cavitary pneumonia who received 6 months of antimicrobial drug treatment had disease relapse (fever, cough, and dyspnea) 9 months after initial presentation. *Rhodococcus* spp. were recovered from bronchoalveolar lavage fluid at relapse. The 4 patients with catheter-associated bacteremia had their catheters removed and did not show relapse.

Conclusions

Our study showed an association between *Rhodococcus* infection and preceding opportunistic infection. This finding suggests that affected patients have a high net state of immunosuppression. Prior cytomegalovirus infection, the most common opportunistic infection in the study, might have had an immunomodulatory effect that made patients more likely to show development of a second opportunistic infection.

Most patients were not neutropenic at diagnosis, consistent with the fact that *Rhodococcus* spp. affect mainly patients with impaired cell-mediated immunity. TMP/SMX prophylaxis did not confer protection, probably because of high resistance rates. Patients did not always have a history of exposure to livestock as previously described (*2*). Median time to infection was shorter for HSCT recipients, probably because catheter-associated bacteremia

Table 2. Univariate analysis of risk factors associated with *Rhodococcus* infection in solid organ and hematopoietic stem cell transplant recipients, United States*

Variable	Case-patients, n = 18	Control patients, n = 36	Univariate OR (95% CI)	p value
Mean age, y (range)	55 (3–78)	50 (2–78)	1.05 (0.99–1.11)	0.13
Male sex	12/18 (66.7)	22/36 (61.1)	1.21 (0.42–3.45)	0.72
White race	15/18 (83.3)	23/36 (63.9)	3.17 (0.65–15.43)	0.15
Diabetes mellitus†	9/18 (50.0)	6/34 (17.6)	9.90 (1.20–81.62)	0.03
Chronic kidney disease‡	3/16 (18.8)	5/35 (14.3)	1.15 (0.19–7.03)	0.88
Immunosuppressant				
Tacrolimus	10/18 (55.6)	25/35 (71.4)	0.15 (0.02–1.39)	0.10
Sirolimus	3/18 (16.7)	2/35 (5.7)	4.65 (0.46–46.89)	0.19
Mycophenolate mofetil	10/18 (55.6)	18/35 (5.4)	1.36 (0.20–9.0)	0.75
Prednisone	13/18 (72.2)	25/35 (71.4)	1.00 (0.25–4.0)	1.00
Cyclosporine	2/18 (11.1)	3/35 (8.6)	2.00 (0.13–31.98)	0.62
Increased calcineurin inhibitor level§	2/13 (15.4)	4/32 (13.3)	1.20 (0.16–9.20)	0.86
History of allograft rejection	2/12 (16.7)	1/24 (4.2)	4.00 (0.36–44.11)	0.26
Augmented immunosuppression¶	7/18 (38.9)	13/37 (35.1)	1.28 (0.24–6.89)	0.77
TMP/SMX prophylaxis	10/18 (55.6)	19/36 (52.7)	1.15 (0.33–4.03)	0.83
History of opportunistic infection#	7/18 (38.9)	4/36 (11.1)	10.57 (1.25–89.0)	0.03

*Values are no. (%) unless otherwise indicated. OR, odds ratio; TMP/SMX, trimethoprim/sulfamethoxazole.
†Requiring treatment with oral antidiabetic agent(s) or insulin.
‡Creatinine level >2 mg/dL.
§Tacrolimus >12 μg/mL or cyclosporine >250 μg/mL in the preceding 30 days.
¶Use of corticosteroid pulses, alemtuzumab, anti-thymocyte globulin, basiliximab, or rituximab in the 6 months preceding infection.
#Opportunistic infections in case-patients were cytomegalovirus viremia (3) or invasive disease (2), pulmonary aspergillosis (1), and BK polyomavirus–associated hemorrhagic cystitis (1).

was more common among these patients. Predominantly among SOT recipients, infection occurred late after transplant (>12 months), and none of the infections were catheter-associated (*2*).

For systemic infections, monotherapy might result in emergence of resistance. In a report from Taiwan, 3 of 7 *R. equi* isolates had inherent concomitant resistance to all β-lactams, macrolides, and rifampin (*9*). We did not observe this multidrug-resistance pattern. For transplant recipients with systemic infection, we recommend combination treatment with 2–3 antimicrobial drugs (vancomycin, fluoroquinolone, or carbapenem). TMP/SMX and clindamycin should be avoided in empiric treatment regimens because of variable rates of susceptibility. Macrolide antimicrobial drugs, except for azithromycin, decrease the metabolism of cyclosporine, tacrolimus, sirolimus, and everolimus. Conversely, rifampin increases the metabolism of these drugs. These interactions should be considered when treating transplant recipients. *Rhodococcus* spp. can also form adherent biofilms (*10*). Thus, removal of central catheters is imperative in the management of infected patients.

We observed only 1 death attributable to infection in an HSCT recipient. This finding differs from an attributable mortality rate of 34.3% in a multicenter study of 67 patients with AIDS (mean CD4 cell count 35/μL) (*11*). The higher mortality rate probably reflects the degree of immunosuppression among patients with advanced HIV infection and close clinical monitoring of transplant recipients, which enables timely management. Death and relapse rates in our series were comparable with those in a review of 30 cases (*2*).

Our retrospective study had inherent limitations related to collection of data. In the analysis, we included SOT and HSCT recipients who differed in underlying disease states and immunosuppression. The study was also limited by the relatively small number of cases. This limitation was also reflected in wide CIs in risk factor analysis. However, we showed that risk factors for *Rhodococcus* infection were diabetes mellitus and recent opportunistic infection. Outcomes were generally favorable after appropriate and timely treatment.

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Mycobacterium tuberculosis Infection among Asian Elephants in Captivity

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Although awareness of tuberculosis among captive elephants is increasing, antituberculosis therapy for these animals is not standardized. We describe *Mycobacterium tuberculosis* transmission between captive elephants based on whole genome analysis and report a successful combination treatment. Infection control protocols and careful monitoring of treatment of captive elephants with tuberculosis are warranted.

Over the past 20 years, recognition of infection and disease caused by *Mycobacterium tuberculosis* in captive elephants and their keepers in the United States and globally has grown (*1*). We describe the diagnosis and treatment of 2 cases of active tuberculosis (TB), separated by 12 years, in 2 Asian elephants in a closed, captive population. In addition, we describe molecular and comparative genomic analysis of *M. tuberculosis* strains cultured from each elephant to investigate transmission.

The Study

In July 1997, the city of Albuquerque, New Mexico, USA, acquired 2 elephants that had been subject to poor conditions in a small traveling circus. Elephant A, a 31-year-old Asian elephant, and elephant B, an 8-year-old African elephant, were quarantined together in an isolated section of the Albuquerque Biopark modified to hold elephants. Quarterly trunk washings (*2*) taken from both elephants over the course of 1 year of quarantine were negative for *M. tuberculosis* by culture. Both elephants were subsequently transferred to the zoo in late 1998.

Trunk washings taken from the zoo's elephant herd every 6 months were negative for *M. tuberculosis* by culture until October 2000, when a specimen from elephant A was found to be positive on 7H11 bi-plates. Elephant A had TB diagnosed and was isolated and monitored, having 3 trunk washings collected over each 7-day period; this cycle continued until week 12 of treatment, when all 3 washings from that week were negative for *M. tuberculosis*. Among the 13 cultured *M. tuberculosis* isolates recovered from 6 different collections, IS*6110* genetic analysis (*3*,*4*) identified 3 different strains (Figure 1). Antimycobacterial susceptibility testing (*5*) revealed that 1 strain was mono-rifampin resistant and all others were pansusceptible.

Initial treatment efforts included an anti-TB regimen of isoniazid (5 mg/kg), rifampin (10mg/kg), and pyrazinamide (25 mg/kg). This regimen was initially given orally, but the administration failed because of elephant's A refusal to ingest the medication despite attempts to disguise or mix the drugs with treats or other food. As a result, isoniazid and pyrazinamide were given rectally, with serum concentrations obtained at 1, 2, and 4 hours after administration. This regimen was continued daily for 2 months, then every other day (QOD) for 1 year (Figure 2). The lengthy 3-month period until trunk washings were negative for *M. tuberculosis* by culture, the use of only 2 drugs, and the switch to a QOD regimen after only 2 months raised concern that elephant A's treatment was suboptimal. In particular, pyrazinamide attacks a specific subpopulation of organisms, and in humans, the selection of drug resistance to other agents in the treatment regimen is increased (*6*).

In response to elephant A's *M. tuberculosis*–positive trunk washings and contact history, elephant B was segregated from the herd and given isoniazid rectally QOD for 6 months as preventive therapy. Both elephants tolerated daily isoniazid poorly, having observable depression and loss of appetite. Clinical signs improved substantially with the start of QOD dosing, and both elephants completed therapy at the end of December 2001.

In December 2010, an Asian elephant (elephant C) had an *M. tuberculosis*–positive serologic test result by

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Chembio DPP VetTB Assay for Elephant (Chembio Diagnostic Systems, Inc., Medford, NY, USA) (*7*). An aggressive trunk washing testing cycle of 3 consecutive days on, followed by 3 days off, was initiated. A specimen from January 2011 produced a single colony of *M. tuberculosis* on 7H11 agar. Genetic analysis revealed an identical IS*6110* fingerprint pattern (Figure 1, lane 6) to the predominant pansusceptible strains isolated from elephant A (Figure 1, lanes 2, 3, and 4). Elephant C's isolate was pansusceptible to all first-line anti-TB drugs. Treatment of elephant C was initiated in May 2011 by published guidelines (*2*); in this case, ethambutol was added to isoniazid and pyrazinamide with the same dosing used to treat elephant A. Routine therapeutic monitoring of serum concentrations led to discontinuing ethambutol and instituting enrofloxacin, a 4-fluoroquinolone used primarily in veterinary settings (*6*). Treatment was completed after 18 months. The previous concern that elephant A had received suboptimal therapy led to her retreatment in concert with elephant C.

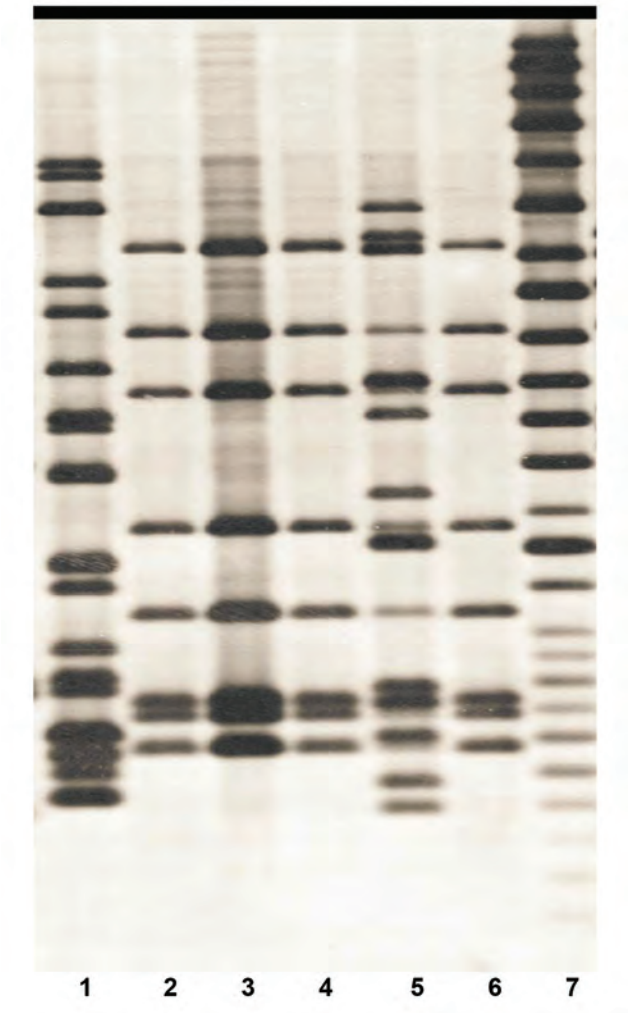

Figure 1. IS*6110* Southern blot hybridization patterns of 6 *Mycobacterium tuberculosis* isolates recovered from elephants A (lanes 1–5) and C (lane 6) (*4*) in study of tuberculosis in captive elephants, Albuquerque, New Mexico, USA, 1997–2013. The fingerprint pattern in lane 1 types the strain to principal genetic group 1, the fingerprint pattern in lanes 2–4 and lane 6 types the strain to principal genetic group 2 and the fingerprint pattern in lane 5 types the strain to principal genetic group 3. Lane 7, molecular mass standard.

A contact investigation was performed among the entire Albuquerque Biopark staff (178 persons) by using tuberculin skin testing and an interferon-gamma release assay (*8*,*9*). No evidence of *M. tuberculosis* transmission was found.

The *M. tuberculosis* isolate recovered from elephant C exhibited an identical IS*6110* fingerprint pattern to the EH3 isolate from elephant A (Figure 1). Although transmission between elephants A and C is not certain, exposure occurred before elephant's A first positive trunk washing, and no coincident TB disease was detected among the Albuquerque Biopark staff. More than 10 years after cohabitation with elephant A in the elephant barn, elephant C had onset of active TB caused by an isolate that had the identical IS*6110* fingerprint to the infecting *M. tuberculosis* isolate from elephant A. Comparative whole genome sequencing analysis of the EH3 *M. tuberculosis* strains from elephants A and C was performed by using an Illumina MiSeq platform (Illumina, Inc., San Diego, CA, USA) (*10*). Single-nucleotide polymorphism (SNP) analysis revealed a total of 3 nt changes (compared with the reference genome H37Rv [GenBank accession no. NC_000962]) at positions 10774 (C to T), 2492143 (C to G), and 3013272 (T to C) during the 11-year period, a rate consistent with the modeled 0.3 SNPs per genome per year as previously reported (*10*,*11*). The sequencing data has been deposited in the National Center for Biotechnology Information database under the BioProject ID no. PRJNA328788.

Recently, whole genome sequencing was used to analyze genetic variation among strains from relapsed patients with recurrent TB (*10*) and to establish genomic mutation rates in a study with nonhuman primates (*11*). Remarkably, both studies showed limited genomic variation and yielded the consensus that *M. tuberculosis* chromosomes change at a rate 0.3 SNPs per year.

Conclusions

Our whole-genome sequencing analysis confirmed the molecular identity of 2 *M. tuberculosis* isolates recovered 12 years apart from 2 captive elephants. The remarkable conservation between the 2 genomes is consistent with previous studies (*10*,*11*). Because elephant C had routine trunk washings that were negative during this period and no intermittent shedding occurred, we believe elephant C had latent TB and that elephant A was the source of the infection

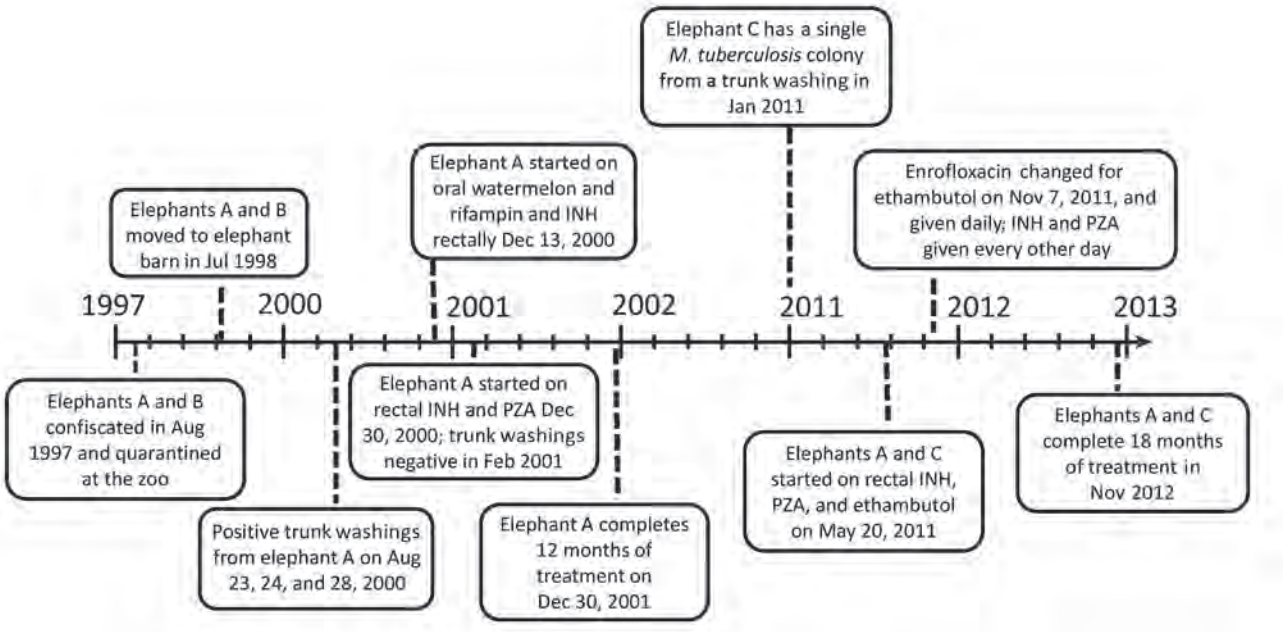

Figure 2. Annotated timeline documenting the course of events in study of tuberculosis in captive elephants, Albuquerque, New Mexico, USA, 1997–2013. INH, isoniazid; PZA pyrazinamide.

during their cohabitation. In support of the probable transmission between elephants A and C, the records at the Albuquerque Biopark confirm that both elephants were barned at the end of the each day from July 1998 through August 2000, when elephant A had a positive trunk washing and was isolated.

Numerous challenges were met in managing the treatment and control of TB among the elephants in the Biopark, including the choice of antimycobacterial agents, their comprehensive delivery, and the appropriate length of treatment. Rectal administration proved the most efficacious, and this approach was adopted for treating elephants A and C and for preventive treatment of elephant B. The availability of enrofloxacin, a fluoroquinolone not used in human health, appeared to be an effective third agent to combine with isoniazid and pyrazinamide, although prospective study data are lacking. This $20,000 regimen (per elephant per year) was ultimately used for 1 year to successfully treat elephant C and to retreat elephant A. The fact that no skin test conversions or TB disease were documented among Biopark staff with epidemiologic association to the elephants supports the evidence that transmission did not involve humans at that location.

Dr. Simpson is an infectious disease physician with experience treating tuberculosis in humans. He is affiliated with the University of New Mexico, Texas Tech University, and the Albuquerque Biopark in Albuquerque, New Mexico, where he was involved in the care of elephants with tuberculosis.

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Molecular Evidence of Drug Resistance in Asymptomatic Malaria Infections, Myanmar, 2015

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Artemisinin resistance containment in Myanmar was initiated in 2011 after artemisinin-resistant *Plasmodium falciparum* malaria was reported. Molecular evidence suggests that asymptomatic malaria infections harboring drug resistance genes are present among residents of the Myanmar artemisinin resistance containment zone. This evidence supports efforts to eliminate these hidden infections.

The global burden of malaria has been decreasing in recent years as a result of high levels of control of the spread of infection, and the ultimate goal of malaria elimination by 2030 in all Greater Mekong Subregion countries in Southeast Asia seems attainable (*1*). However, artemisinin-resistant *Plasmodium falciparum* malaria has been reported in Cambodia, Thailand, Myanmar, Laos, and Vietnam (*2*). Chloroquine-resistant *P. vivax* malaria has also been confirmed in 10 countries, including Myanmar (*3*), and mutations in the mefloquine-resistance molecular marker (*pvmdr1* mutation) and sulfadoxine/pyrimethamine-resistance markers (*pvdhps, pvdhfr* mutations) have been reported in Myanmar (*4*).

A containment program for artemisinin-resistant malaria was initiated in 2011 according to the Global Plan for Artemisinin Resistance Containment. Areas where artemisinin resistance was documented were ranked as Tier I under the protocol, whereas areas where resistance was suspected were ranked as Tier II. After Myanmar artemisinin resistance containment (MARC) was initiated, malaria morbidity and mortality rates decreased dramatically, especially in MARC Tier I areas (*5*). However, there are no reports on the prevalence of asymptomatic infections, which may represent a reservoir of local malaria transmission. In this study, we aimed to determine the prevalence of asymptomatic malaria infection and to analyze drug-resistance markers in asymptomatic *P. falciparum* and *P. vivax* infections.

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The Study

As of 2014, the Tier I area of artemisinin resistance in Myanmar was composed of 52 townships; the remaining regions were designated as Tier II. In January 2015, we conducted a cross-sectional study of one of the Tier I areas of the MARC, Shwegyin Township (22°20′0″N, 95°56′0″E) (Figure; online Technical Appendix, https://wwwnc.cdc.gov/EID/article/23/3/16-1363-Techapp1.pdf).

Rapid diagnostic tests (RDTs) (HRP2 and *P. vivax*–specific pLDH-based RDT, SDFK80; Standard Diagnostics, Gyeonggi-do, South Korea), microscopy, and PCR were used to screen for asymptomatic malaria infection (online Technical Appendix). We examined 1,182 local residents, with a male:female ratio of 4:5 and a median age of 30 years (interquartile range 18–45 years). Among these residents, 549 (46.4%) had a history of malaria infection within the past 5 years. No clinical cases of malaria infection were detected during the survey period. Although we found no RDT-positive cases of malaria infection, we detected 2 *P. vivax* infections by microscopy, with parasite densities of 580 and 1,200 parasites/μL.

When we performed molecular detection for the 4 common malaria species (online Technical Appendix), the overall rate of asymptomatic malaria infection was 2.4% (28/1,180) and included 4 *P. falciparum*, 22 *P. vivax*, and 2 *P. malariae* infections. Although the overall prevalence of asymptomatic infection in these areas was not high, it was similar to that observed in the Thailand–Myanmar border area during 2013–2014 (*6*).

In this study, RDT and microscopy missed almost all the asymptomatic infections detected by PCR, indicating that only the molecular method is suitable for the detection of asymptomatic infections. Moreover, the asymptomatic cases were broadly distributed geographically throughout the study area. Most of the infections were in male patients (19/28, 67.8%) and in the working-age group. Neither sex nor occupation was identified as an associated factor for asymptomatic infection (online Technical Appendix Table 2).

The established artemisinin-resistance marker K13 (kelch 13 gene) and the associated markers *pfarps10* (*P. falciparum* apicoplast ribosomal protein S10), *pffd* (*P. falciparum* ferredoxin), and *pfmdr2* (*P. falciparum* multidrug-resistance protein 2) were analyzed in all asymptomatic

[1]These authors contributed equally to this article.

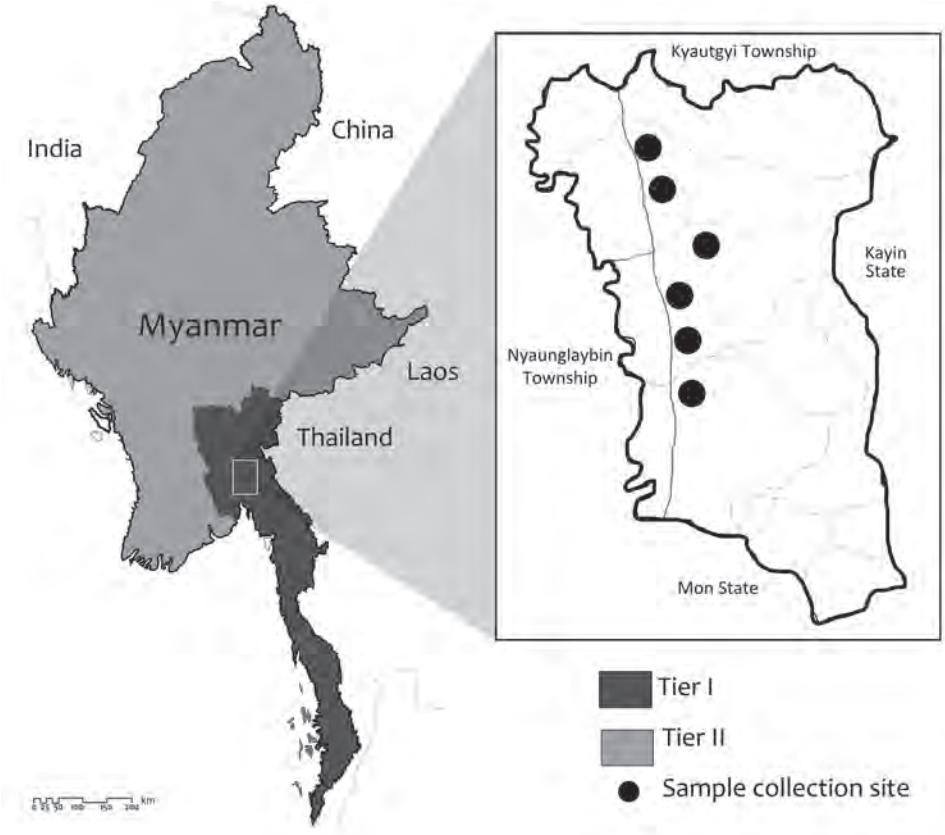

Figure. The study site, Shwegyin Township, Myanmar, where molecular evidence of drug resistance in asymptomatic malaria infections was obtained. As of 2014, Myanmar artemisinin resistance containment areas were divided into Tier I (52 townships) and Tier II (all remaining townships).

P. falciparum cases. Nonsynonymous mutations in the propeller region of K13 were found to be associated with artemisinin resistance and associated delayed clearance of the parasite beyond 72 hours after treatment with artemisinin-based combination therapy (*7*). A previous study in the same region of patients with uncomplicated *P. falciparum* malaria indicated that 25.3% carried mutant K13 alleles (*8*). Markers that showed the underlying genetic background predisposing to the K13 mutant were also reported, including *pfarps10, pffd, pfmdr2*, and *pfcrt*. Specific single nucleotide polymorphisms of these genes, such as V127M of *pfarps10*, D193Y of *pffd*, and T484I of *pfmdr2*, were found at a similar prevalence as K13 mutations (*9*).

Among the 4 asymptomatic *P. falciparum* infections, 2 isolates showed K13 mutations (C580Y in 1 isolate and P574L in the other). C580Y is a well-known validated mutation, and P574L is a candidate marker for artemisinin resistance. Both mutations were reported only in locations in Southeast Asia where artemisinin resistance has been identified (*2*). Moreover, we observed the *pfarps10* mutation (V127M) in 2 of the cases, the *pffd* mutation (D195Y) in 3 cases, and the *pfmdr2* (T484I) mutation in all 4 isolates (Table; online Technical Appendix Table 3). This molecular evidence suggests the presence of artemisinin resistance in asymptomatic isolates and calls for action toward eliminating this parasite reservoir.

Similarly, we analyzed all available drug-resistance molecular markers in *P. vivax* (*10*), such as *pvcrt* (*P. vivax* chloroquine-resistance transporter), *pvdhps* (*P. vivax* dihydropteroate synthase), *pvdhfr* (*P. vivax* dihydrofolate reductase), and *pvmdr1* (*P. vivax* multidrug-resistance protein 1), in all *P. vivax* infections. We conducted analysis by using nested PCR, followed by gene sequencing (online Technical Appendix).

Among the 22 asymptomatic *P. vivax* infections, we were unable to amplify *pvcrt-o, pvdhfr*, and *pvmdr1* in 1 isolate and *pvdhps* in 2 isolates. A high mutation rate was observed in known drug-resistance markers such as *pvcrt-o* K10 AAG insert (66.6%, 14/21), *pvdhps* (100.0%, 20/20), *pvdhfr* (100.0%, 21/21), and *pvmdr1* (100.0%, 21/21) (Table). Asymptomatic isolates in this study showed a higher mutation rate of the *pvcrt-o* AAG insert than those studied in neighboring countries such as Thailand (*11*), India (*12*), and China (*10*). In the *pvmdr1* gene, both Y976F and F1076L mutations were observed in 23.8% of cases and F1076L in 19.0% of cases; these rates were higher than those for China (*10*) and India (*12*) but lower than those for Thailand (*13,14*). Although antifolates are not the recommended antimalarial drugs for treatment of *P. vivax*, *pvdhfr* and *pvdhps* mutation rates were noticeable. This finding indicates that drug pressure in *P. vivax* malaria contributing to drug resistance also

Table. *Plasmodium falciparum* and *P. vivax* drug-resistance molecular markers in asymptomatic infections, Myanmar, 2015

Target	Description*	No. isolates/total (%)
kelch 13 (K13)	Wild	2/4 (50.0)
	C580**Y**	1/4 (25.0)
	P574**L**	1/4 (25.0)
P. falciparum apicoplast ribosomal protein S10 (*pfarps10*)	Wild	2/4 (50.0)
	V127**M**	2/4 (50.0)
P. falciparum ferredoxin (*pffd*)	Wild	1/4 (25.0)
	D193**Y**	3/4 (75.0)
P. falciparum multidrug-resistance protein 2 (*pfmdr2*)	Wild	0/4 (0.0)
	T484**I**	4/4 (100.0)
P. vivax chloroquine-resistance transporter (*pvcrt-o*)	Wild	7/21 (33.3)
	Mutant (AAG insert)	14/21 (66.7)
P. vivax multidrug-resistance protein 1 (*pvmdr1*)	Wild (T, Y, F) (958, 976, 1076)	0/21 (0.0)
	Double mutant (**M**,Y, **L**)	4/21 (19.0)
	Single mutant (**M**, Y, F)	12/21 (57.1)
	Triple mutant (**M**, **F**, **L**)	5/21 (23.8)
P. vivax dihydropteroate synthase (*pvdhps*)	Wild (S, A, K, A) (382, 383, 512, 553)	0/20 (0.0)
	Single mutant (S, **G**, K, A)	4/20 (20.0)
	Double mutant (S, **G**, K, **G**)	9/20 (45.0)
	Triple mutant (**A**, **G**, K, **G**)	5/20 (25.0)
	Quadruple mutant (**A**, **G**, **M**, **G**)	2/20 (10.0)
P. vivax dihydrofolate reductase (*pvdhfr*)	Wild (F, S, T, S) (57, 58, 61, 117)	0/21 (0.0)
	Single mutant (**L**, S, T, S)	1/21 (4.8)
	Double mutant (F, **R**, T, **N**)	2/21 (9.5)
	Quadruple mutant (**L/I**, **R**, **M**, **T**)	18/21 (85.7)

*Numbers in parentheses indicate the amino acid position. Mutant amino acids are shown in bold. All sequences were aligned with 3D7 (*P. falciparum*) and Sal-1 (*P. vivax*) reference sequences from http://www.plasmodb.org.

needs to be considered in addition to emphasizing the artemisinin-resistant *P. falciparum* malaria.

One limitation of this study is the exclusive focus on the local residents in the MARC area, where all available control and prevention measures had already been implemented. Unlike the mobile and migrant population, local residents have not been a top priority for the artemisinin resistance containment program, leading to a niche of hidden infection. Moreover, blood pooling before DNA extraction was used in this study for molecular detection of malaria infection. Although this method is not ultrasensitive, it has a higher sensitivity than RDT and microscopy. The hidden asymptomatic infections and associated molecular markers for drug resistance among the asymptomatic cases detected in this study represent a threat to containment and elimination efforts with regard to drug-resistant parasites.

Conclusions

All countries in the Greater Mekong Subregion have set an ultimate goal of eliminating malaria by 2030. One of the main challenges to achieving this goal is hidden asymptomatic infection, which maintains a reservoir for local transmission of malaria (*15*). Critically, these asymptomatic infections may carry drug-resistance genes, including genes for artemisinin resistance. Our results indicated that drug-resistant malaria parasites may be spreading, even in the containment areas or (pre-)elimination areas; this issue should, therefore, be addressed at a policy level. Detection and elimination of asymptomatic infections are of vital importance. Our evidence highlights the need for a strategy for eliminating drug-resistant malaria in asymptomatic infections in the containment areas.

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Pneumonic Plague Transmission, Moramanga, Madagascar, 2015

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During a pneumonic plague outbreak in Moramanga, Madagascar, we identified 4 confirmed, 1 presumptive, and 9 suspected plague case-patients. Human-to-human transmission among close contacts was high (reproductive number 1.44) and the case fatality rate was 71%. Phylogenetic analysis showed that the *Yersinia pestis* isolates belonged to group q3, different from the previous outbreak.

Plague, caused by the bacterium *Yersinia pestis*, is a fleaborne disease responsible for the death of tens of millions of persons throughout history (*1*,*2*). The bacterium spread to Madagascar in 1898 through trade routes and became endemic in the country's central highlands (*3*). Despite considerable control efforts, *Y. pestis* remains a public health threat in Madagascar (*4*–*6*). For the past 2 decades, Madagascar reported the highest number of human infections in the world (*7*).

Bubonic plague is the most common disease form resulting from the bite of an infected flea. As bubonic plague worsens, it can progress to pneumonic plague, in which persons expel infectious, aerosolized respiratory droplets. During pneumonic plague outbreaks, person-to-person transmission facilitates the spread from the initial infected person to family members and the wider community (*5*,*8*). Pneumonic plague is rare but poses a substantial danger in Madagascar because of the country's relatively weak healthcare system (*4*,*6*) and widespread traditional burial methods involving interaction with corpses. Understanding how pneumonic plague outbreaks spread is vital for managing disease prevention and educating the population. We investigated the transmission chain of a pneumonic plague outbreak that occurred in Madagascar outside the normal plague season (October–March) (*9*) in a remote area that had been free of human plague for 13 years.

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The Study

Over a 10-day period, 14 persons became ill with pneumonia and fever. The first case-patient (case-patient 1) displayed symptoms on August 17, 2015, while in a remote area in Antsahatsianarina hamlet, Tsiazompody village, Ampasipotsy Gara commune, within the Moramanga district of Madagascar (online Technical Appendix Table, https://wwwnc.cdc.gov/EID/article/23/3/16-1406-Techapp1.pdf). Case-patient 1 was a 22-year-old man who had traveled 1 week before disease onset. After returning home, he experienced chest pain, fever, and cough. Two days later, his condition deteriorated and, while relatives and neighbors assisted him to the nearest health center, he died near Beravina, Madagascar. He was buried near Beravina in a traditional manner with a 2-night wake, exposing the family and community to the pathogen and initiating a chain of transmission. On August 22 and 23, plague symptoms developed in members of case-patient 1's immediate family (N = 2), his extended family (N = 6), and the community (N = 3). Of these, 4 died, and 7 self-referred to the Moramanga district hospital, where 4 died upon arrival, and 3 survived after a 6- to 9-day course of antimicrobial drug treatment. On August 25, a second chain of transmission (N = 2) was identified (Figure 1). One case-patient was from the outbreak epicenter, and the other was a suspected nosocomial transmission; no connection to the other cases could be found except occupying the same hospital space as some of the earlier case-patients. Transmission ceased with these last 2 case-patients.

Before illness onset, case-patients 2–14 had social or spatial contact with a symptomatic person who could be traced back to case-patient 1. Two of the deceased case-patients (2 and 10) were visitors to Antsahatsianarina who had returned home to their respective communities Ambatoharanana and Ambilona (Figure 2), potentially spreading the bacterium to others. To investigate the extent of pathogen spread, we identified 123 case-patient contacts in 4 communities connected with case-patient 1 (online Technical Appendix Table). On August 24, 2015, an outbreak investigation protocol was applied by the Institut Pasteur de Madagascar and the Malagasy Ministry of Health, whose ethics committee approved the study (068-MSANP/CE). Verbal consent was obtained from 71 contacts to test their serum for plague antibody with a capsular antigen fraction 1 (F1) IgG ELISA (*10*,*11*); 7/20 (35%) contacts from Antsahatsianarina, 12/20 (60%) from Beravina (the

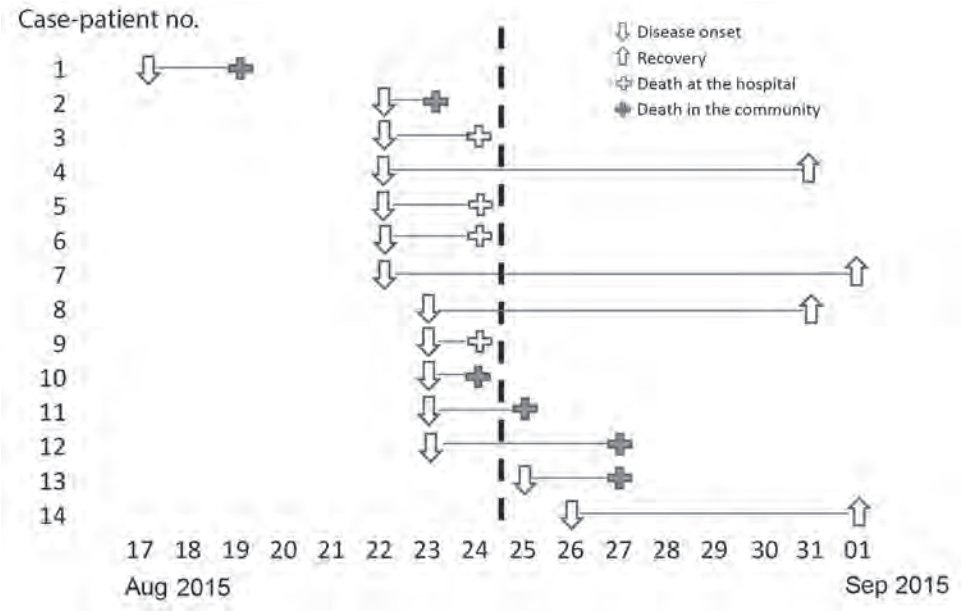

Figure 1. Course of pneumonic plague outbreak in Moramanga, Madagascar, August 17–September 1, 2015 (N = 14). Each line corresponds to a case-patient and describes disease outcomes. The vertical dashed line denotes when control measures began.

burial site of case-patient 1), 9/10 (90%) from Ambilona, and 7/21 (33%) from Ambatoharanana were seropositive (Figure 2; online Technical Appendix Table). The 35 contacts positive for F1-specific antibody were given chemoprophylaxis, and their infections remained subclinical.

The 14 case-patients who had sought medical care for pneumonia and fever had clinical signs and symptoms consistent with those for plague. To confirm *Y. pestis* infection, we performed bacteria culture on sputum or organ puncture (*12*) and serologic testing of serum samples (*10*). According to the international standards definition (*13*), 4/5 patients (1 deceased) were confirmed positive for *Y. pestis* (2 by culture and 2 by seroconversion) and 1/5 was presumptive for *Y. pestis* infection. No samples were collected from the other 9 (all deceased) persons, who were thus considered suspected plague case-patients (*13*).

More men (n = 10) than women (n = 4) were infected. Median patient age was 22.5 (range 15–80) years, and the overall case-fatality rate was 71%. The most common signs and symptoms were fever, dyspnea, chest pain, and cough (100% of patients); blood-stained sputum (93%); chills (86%); and headache (71%). The average duration from onset of full-blown disease to death was 1.9 days, and the average infectious period was 3.5 days. Basic reproductive

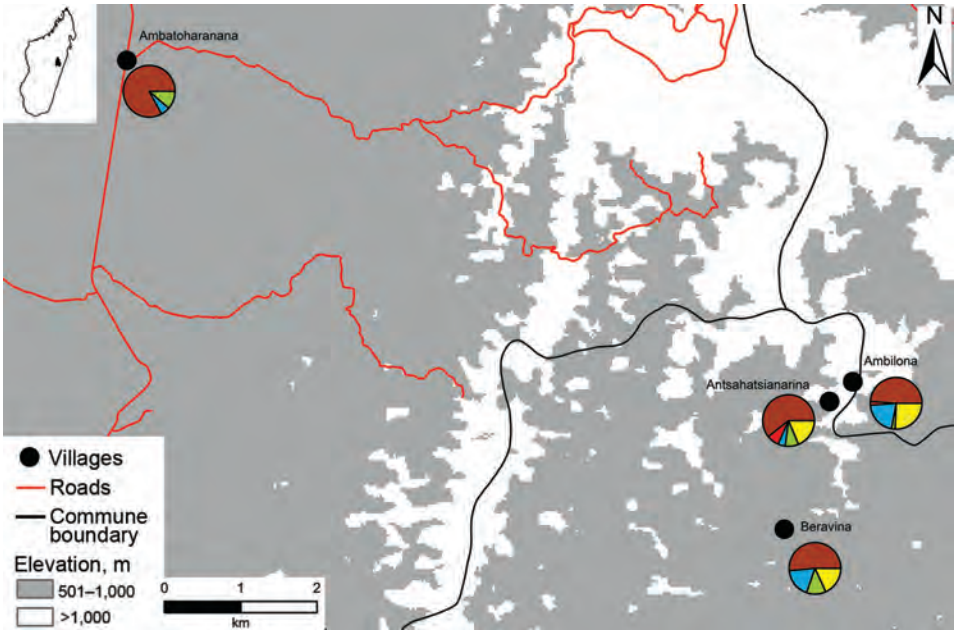

Figure 2. Map of pneumonic plague outbreak (n = 14) in the commune of Ampasipotsy Gara in Moramanga, Madagascar, 2015. The index case-patient (case-patient 1) was infected with *Yersinia pestis* at Antsahatsianarina and spread the bacterium to Beravina (burial site of case-patient 1), Ambilona (case-patient 10's home), and Ambatoharanana (burial site of case-patient 2 and home of case-patient 14). Each pie chart indicates the proportions of plague cases (red), seropositive contacts (blue), seronegative contacts (green), unsampled contacts (yellow), and noncontacts (brown) among the total inhabitants of each site.Pie chart details are given in the online Technical Appendix (https://wwwnc.cdc.gov/EID/article/23/3/16-1406-Techapp1.pdf).

number (R_0) was 1.44, and the transmission rate was 0.41 susceptibles/day (*14*).

To investigate the possible outbreak origin, we trapped 100 rats and mice over a period of 3 nights at Antsahatsianarina, Beravina, and Ambilona. Rodent spleens were sampled in accordance with the directive 2010/63/EU of the European Parliament (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:276:0033:0079:EN:PDF) and tested by using an F1 antigen rapid diagnostic test (*3*). A total of 22 (22%) rats, but no mice, from the 3 communities tested positive for plague antigen (Table).

The lack of human plague activity in this area for the past 13 years suggests that a reservoir species, such as rodents or fleas, transmitted bubonic plague to case-patient 1 and that the disease later progressed into the pneumonic form. Relatives of case-patient 1 indicated that moribund rodents were present near his home 2 weeks before he became ill. The family who hosted case-patient 1 during his travels (1 week before his disease onset) reported no disease present in their household. Taken together, the epidemiologic data suggest that case-patient 1's hamlet (Antsahatsianarina) was the probable outbreak epicenter.

Using published single-nucleotide polymorphisms (*15*), we assigned isolates from 2 case-patients and 1 recently sampled rat to the *Y. pestis* q3 phylogenetic subgroup within group I (node k). The matched genetic grouping between the 2 human samples is consistent with human-to-human transmission. The matched genetic grouping among all 3 samples is consistent with *Y. pestis* in the initial case-patient originating from the environment rather than another human. We compared these phylogenetic data with data from 4 archived isolates (human and rodent) obtained in 2000, 2002, and 2003 from the same area. The archived isolates were also assigned to group I (node k), but unlike the isolates from this study, they were assigned to the q2 subgroup (*15*). This finding indicates that the recent outbreak did not arise from the same phylogenetic groups responsible for past outbreaks and illustrates how outbreaks in different years are probably conferred by *Y. pestis* from different environmental sources. As of January 2017, 4 lineages (q1, q2, q3, and q4) have been recorded in Moramanga, suggesting multiple genotypes are persisting within this region (*15*).

Table. Number of *Yersinia pestis* antigen–positive rodents in a study of pneumonic plague transmission among humans, Moramanga, Madagascar, 2015*

Locality and species	No. samples	No. RDT positive	No. RDT negative
Antsahatsianarina			
Rattus rattus	73	16	57
Rattus norvegicus	10	1	9
Mus musculus	4	0	4
Beravina			
Rattus rattus	4	3	1
Rattus norvegicus	0	0	0
Mus musculus	2	0	2
Ambilona			
Rattus rattus	6	2	4
Rattus norvegicus	1	0	1
Mus musculus	0	0	0
Total	100	22	78

*A rapid diagnostic test (RDT) was used to test for *Yersinia pestis* antigen.

Conclusions

Despite Madagascar having an effective surveillance system, plague control remains a public health challenge. Pneumonic plague is rare but persists as a threat in Madagascar, where poor healthcare systems and traditional burial practices promote these outbreaks. Our findings provide additional understanding of pneumonic plague transmission patterns, argues for continued public education, and informs authorities about effective outbreak response practices.

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Outbreaks of Disease Associated with Food Imported into the United States, 1996–2014[1]

L. Hannah Gould,[2] Jennifer Kline, Caitlin Monahan, Katherine Vierk

The proportion of US food that is imported is increasing; most seafood and half of fruits are imported. We identified a small but increasing number of foodborne disease outbreaks associated with imported foods, most commonly fish and produce. New outbreak investigation tools and federal regulatory authority are key to maintaining food safety.

Approximately 19% of food consumed in the United States is imported, including ≈97% of fish and shellfish, ≈50% of fresh fruits, and ≈20% of fresh vegetables (*1*). The proportion of food that is imported has increased steadily over the past 20 years because of changing consumer demand for a wider selection of food products and increasing demand for produce items year round (*1*).

The Centers for Disease Control and Prevention (CDC) defines a foodborne disease outbreak as the occurrence of ≥2 persons with a similar illness resulting from ingestion of a common food (*2*). Local, state, and territorial health departments report foodborne disease outbreaks to CDC through the Foodborne Disease Outbreak Surveillance System. The information collected for each outbreak includes etiology (confirmed or suspected on the basis of predefined criteria) (*2*), year, month, state, implicated food, and number of illnesses, hospitalizations, and deaths. Information is also collected on where implicated food originated. During 1973–1997, this information was reported anecdotally in the report's comments section. During 1998–2008, "contaminated food imported into U.S." was included as a location where food was prepared. Since 2009, the form has included a variable to indicate whether an implicated food was imported into the United States and the country of origin.

The Study

We reviewed outbreak reports to identify outbreaks associated with an imported food from the inception of the surveillance system in 1973 through 2014, the most recent year for which data were available. We obtained additional data for some outbreaks (e.g., country of origin) from the US Food and Drug Administration (FDA) and the US Department of Agriculture Food Safety and Inspection Service.

We categorized implicated foods by using the schema developed by the Interagency Food Safety Analytics Collaboration (*3*). We grouped countries using the United Nations Statistics Division classification (*4*). We conducted a descriptive analysis of the number of outbreaks over time, by food category, and by region of origin.

During 1996–2014, a total of 195 outbreak investigations implicated an imported food, resulting in 10,685 illnesses, 1,017 hospitalizations, and 19 deaths. Outbreaks associated with imported foods represented an increasing proportion of all foodborne disease outbreaks where a food was implicated and reported (1% during 1996–2000 vs. 5% during 2009–2014). The number of outbreaks associated with an imported food increased from an average of 3 per year during 1996–2000 to an average of 18 per year during 2009–2014 (Figure).

The most common agents reported in outbreaks associated with imported foods were scombroid toxin and *Salmonella*; most illnesses were associated with *Salmonella* and *Cyclospora* (Table). Aquatic animals were responsible for 55% of outbreaks and 11% of outbreak-associated illnesses. Produce was responsible for 33% of outbreaks and 84% of outbreak-associated illnesses. Outbreaks attributed to produce had a median of 40 illnesses compared with a median of 3 in outbreaks attributed to aquatic animals. All but 1 of the outbreaks caused by scombroid toxin was associated with fish. Most of the *Salmonella* outbreaks (77%) were associated with produce, including fruits (n = 14), seeded vegetables (n = 10), sprouts (n = 6), nuts and seeds (n = 5), spices (n = 4), and herbs (n = 1).

Information was available on the region of origin for 177 (91%) outbreaks. Latin America and the Caribbean was the most common region implicated, followed by Asia (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/23/3/16-1462-Techapp1.xlsx). Thirty-one countries were implicated; Mexico was most frequently implicated (42 outbreaks). Other countries associated with >10 outbreaks were Indonesia (n = 17) and Canada (n = 11). Fish and shellfish originated from all regions except Europe but

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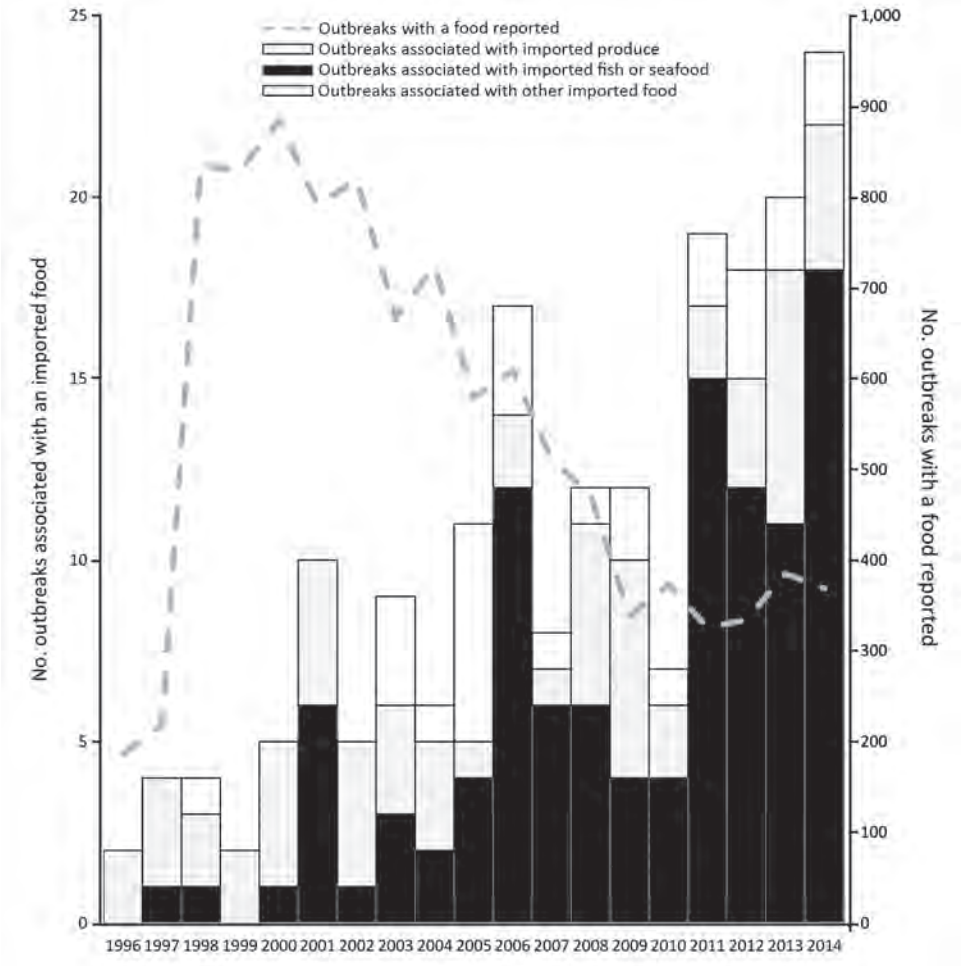

Figure. Number of outbreaks caused by imported foods and total number of outbreaks with a food reported, United States, 1996–2014. Reporting practices changed over time; 1973–1997, imported foods anecdotally noted in report comments; 1998–2008, "contaminated food imported into U.S." included as a location where food was prepared; 2009–2014, reporting jurisdictions could indicate whether each food is imported (yes/no) and the country of origin.

were most commonly imported from Asia (65% of outbreaks associated with fish or shellfish). Produce originated from all regions but was most commonly imported from Latin America and the Caribbean (64% of outbreaks associated with produce). All but 1 outbreak associated with dairy products involved products imported from Latin America and the Caribbean.

Outbreaks in this analysis were reported from 31 states, most commonly California (n = 30), Florida (n = 25), and New York (n = 16). Forty-three outbreaks (22%) were multistate outbreaks.

Conclusions

The number of reported outbreaks associated with imported foods, although small, has increased as an absolute number and in proportion to the total number of outbreaks in which the implicated food was identified and reported. Although many types of imported foods were associated with outbreaks, fish and produce were most common. These findings are consistent with overall trends in food importation (*5*).

Many outbreaks, particularly outbreaks involving produce, were associated with foods imported from countries in Latin America and the Caribbean. Because of their proximity, these countries are major sources of perishable items such as fresh fruits and vegetables; Mexico is the source of about one quarter of the total value of fruit and nut imports and 45%–50% of vegetable imports, followed by Chile and Costa Rica. Similarly, our finding that many outbreaks were associated with fish from Asia is consistent with data on the sources of fish imports (*6*).

One quarter of the outbreaks were multistate, reflecting the wide distribution of many imported foods. Systems like PulseNet have helped to improve detection and investigation of multistate outbreaks, resulting in an increased number of multistate outbreaks (*7*,*8*). The increasing number of outbreaks involving globally distributed foods underscores the need to strengthen regional and global networks for outbreak detection and information sharing. The importance of having standard protocols for molecular characterization of isolates and systems for rapid traceability of implicated foods to their source was illustrated during the investigation of a listeriosis outbreak linked to Italian cheese imported into the United States in 2012 (*9*). Newer tools like whole genome sequencing can also help to generate

Table. Outbreaks and illnesses caused by imported foods, by causative agent and food category, United States, 1996–2014*

Etiology	No. (%) Outbreaks	No. (%) Illnesses
Agent		
Scombroid toxin	57 (31)	192 (2)
Salmonella	52 (28)	4,421 (42)
Ciguatoxin	18 (10)	76 (0.7)
Cyclospora	11 (6)	3,533 (33)
Norovirus	10 (5)	131 (1)
Escherichia coli O157	6 (3)	116 (1)
Shigella sonnei	5 (3)	625 (6)
Vibrio parahaemolyticus	5 (3)	243 (2)
Listeria monocytogenes	4 (2)	67 (0.6)
Hepatitis A virus	4 (2)	1150 (11)
Brucella	3 (2)	11 (0.1)
Other†	9 (5)	38 (0.4)
Food category		
Aquatic animals		
Fish	88 (45)	830 (8)
Mollusks	17 (9)	350 (3)
Crustaceans	1 (0.5)	18 (0.2)
Other seafood	1 (0.5)	14 (0.1)
Land animals		
Dairy	12 (6)	140 (1)
Beef	1 (0.5)	29 (0.3)
Eggs	1 (0.5)	58 (0.5)
Game	1 (0.5)	2 (0)
Produce		
Fruits	22 (112)	3,450 (32)
Seeded vegetables	11 (6)	1,847 (17)
Sprouts	10 (5)	510 (5)
Vegetable row crops	7 (4)	1,241 (12)
Spices	4 (2)	530 (5)
Herbs	4 (2)	1,147 (11)
Other produce	2 (1)	154 (1)
Other plants		
Nuts and seeds	5 (3)	132 (1)
Oils and sugars	2 (1)	10 (0.1)
Grains and beans	1 (0.5)	89 (0.8)
Multiple etiology‡	5 (3)	134 (1)

*Causative agent data were available for 184 outbreaks involving 10,603 illnesses. Food category data were available for 195 outbreaks involving 10,685 illnesses.
†Other agents implicated were tetrodotoxin (3 outbreaks) and *Campylobacter*, chaconine, *Paragonimus,* other virus, sulfite, and *Trichinella* (1 outbreak each).
‡Foods implicated were a chicken dish, crab cake, creampuff, beer, and a wheat snack (1 outbreak each).

hypothetical transmission networks and in some instances facilitate traceback of foods to their origin (*10*). Moreover, new tools that aid visualization of supplier networks facilitate the investigation of outbreaks involving the increasingly complex global economy (*11*).

Nearly all of the outbreaks involved foods under FDA jurisdiction. Only a small proportion of FDA-regulated foods are inspected upon entry into the United States. New rules under the Food Safety Modernization Act of 2011, including the Preventive Controls Rule for Human Food, Produce Safety Rule, Foreign Supplier Verification Program, and Accreditation of Third Party Auditors, will help to strengthen the safety of imported foods by granting FDA enhanced authorities to require that imported foods meet the same safety standards as foods produced domestically (*12*).

Although data collection has improved in recent years, these findings might underestimate the number of outbreaks associated with imported foods because the origin of only a small proportion of foods causing outbreaks is reported. Similarly, because of how data are collected and reported, the relative safety of imported and domestically produced foods cannot be compared. Because of changes in surveillance and changing import patterns, changes over time should be interpreted cautiously.

Our findings reflect current patterns in food imports and provide information to help guide future outbreak investigations. Prevention focused on the most common imported foods causing outbreaks, produce and seafood, could help prevent outbreaks. Efforts to improve the safety of the food supply can include strengthening reporting by gathering better data on the origin of implicated food items, including whether imported and from what country.

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Zoonotic Transmission of *mcr-1* Colistin Resistance Gene from Small-Scale Poultry Farms, Vietnam

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We investigated the consequences of colistin use in backyard chicken farms in Vietnam by examining the prevalence of *mcr-1* in fecal samples from chickens and humans. Detection of *mcr-1*–carrying bacteria in chicken samples was associated with colistin use and detection in human samples with exposure to *mcr-1*–positive chickens.

Colistin resistance is a gradually emerging problem among gram-negative bacteria in clinical settings in many countries (*1*). A transferable plasmid-derived colistin resistance gene *mcr-1* discovered in China and subsequently found worldwide could be mediating this emergence (*2*,*3*). Use of colistin in animal production has been suggested as the most likely factor contributing to the emergence of the *mcr-1* gene (*2*). However, systematic studies applying the One Health approach to investigate the epidemiologic link between the use of colistin in agriculture and colonization with *mcr-1*–carrying bacteria in the community are lacking (*4*).

Colistin use in humans is negligible (*5*), but it is one of the most commonly used antimicrobial drugs in animal production in Vietnam (*6*). We investigated the consequences of colistin use in chicken farms by assessing chickens, farmers, and nearby persons for the presence of *mcr-1*–carrying bacteria and performing epidemiologic analyses to assess the risk for subsequent transmission to unexposed human populations in southern Vietnam.

The Study

From March 2012 to April 2013, we conducted a systematic, cross-sectional study examining antimicrobial drug use and colonization with antimicrobial-resistant *E. coli* in chickens and humans in Tien Giang Province, Vietnam. Fecal samples from 204 chicken farms and rectal swabs from 204 chicken farmers (1 farmer/farm) were collected as described (online Technical Appendix 1, https://wwwnc.cdc.gov/EID/article/23/3/16-1553-Techapp1.pdf) (*7*,*8*). We additionally collected rectal swabs from age- and sex-matched persons not involved in poultry farming from the same districts (rural persons, n = 204) and from their provincial capitals (urban persons, n = 102) (*8*).

Samples were cultured on MacConkey plates with and without antimicrobial drugs. A sweep of the full growth on plain MacConkey plates was collected and screened for the presence of *mcr-1* by PCR as described previously (*2*). Logistic regression models were built to investigate the risk factors associated with the presence of *mcr-1* on chicken farms and in human participants. Then, we selected (using a random number table) individual *E. coli* colonies (n = 200) and extended-spectrum β-lactamase (ESBL)–producing *E. coli* colonies (n = 122) growing on different MacConkey plates and repeated PCR to confirm the presence of *mcr-1* in *E. coli* isolated from chickens and humans. We tested all *mcr-1*–positive *E. coli* isolates for colistin susceptibility using Etest (bioMérieux, Marcy l'Etoile, France) and interpreted test results in accordance with the European Committee on Antimicrobial Susceptibility Testing breakpoints (*9*). In addition, whole-genome sequencing was performed on all *mcr-1*–positive *E. coli* isolates as described (online Technical Appendix 1).

From a total of 204 chicken and 510 human fecal specimens, 188 and 440 MacConkey sweeps were available for *mcr-1* screening by PCR, respectively. The adjusted prevalence of *mcr-1* was 59.4% (95% CI 47.9%–71.0%) in chicken and 20.6% (95% CI 15.9%–25.2%) in human fecal samples (Table 1).

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Table 1. Prevalence of fecal colonization with *mcr-1*–carrying bacteria in chickens and humans, Tien Giang Province, Vietnam, 2012–2013

Source	Prevalence of fecal colonization with *mcr-1*–carrying bacteria	
	No. positive sweeps/total (%)	Adjusted prevalence, % (95% CI)
All chicken farms	93/188 (49.5)	59.4 (47.9–71.0)
Household chicken farms	53/94 (56.4)	59.5 (47.9–71.1)
Small-scale chicken farms	40/94 (42.6)	47.9 (35.4–60.3)
All human participants	84/440 (19.1)	20.6 (15.9–25.2)
All farmers	45/179 (25.1)	25.2 (18.3–32.0)
Farmers exposed to *mcr-1*–negative chickens	16/91 (17.6)	15.5 (7.7–23.3)
Farmers exposed to *mcr-1*–positive chickens	29/88 (33.0)	34.7 (23.9–45.5)
Rural persons	31/173 (17.9)	17.6 (11.6–23.7)
Urban persons	8/88 (9.1)	9.1 (3.1–15.1)

Among 200 *E. coli* isolates, *mcr-1* was detected in 10/78 (12.8%) isolates from chickens, 2/50 (4.0%) isolates from farmers, and 0/72 isolates from persons who did not farm. Similarly, *mcr-1* was detected in 9/38 (23.7%) and 1/44 (2.3%) of ESBL-producing *E. coli* isolated from chickens and farmers, respectively.

The MIC of colistin for the 22 *mcr-1*–carrying *E. coli* isolates ranged 3–4 mg/L. Because the Etest might underestimate the true MIC (*10*), these results indicate reduced susceptibility. Single-nucleotide polymorphism (SNP)–based phylogenetic analyses of the core genomes showed little genomic similarity between isolates, but the analyses did show many isolates belonged to the same multilocus sequence types (n = 14) (Figure). Analysis of the acquired resistance genes, reflecting the presence of an accessory genome, showed a large variation in resistance gene content, with only the *tet*(A) gene, encoding for tetracycline resistance, present in all genomes (online Technical Appendix 2 Table, https://wwwnc.cdc.gov/EID/article/23/3/16-1553-Techapp1.xlsx). De novo bacterial genome assembly was performed, and the contigs carrying *mcr-1* were analyzed. A replication origin could be located in 5 isolates, leading to the identification of plasmid incompatibility groups IncHI2 (1 isolate), IncI2 (2 isolates), and combined IncHI2 and IncHI2A (2 isolates). Transposon IS*AplI*, initially described as carrying the *mcr-1* gene (*2*), was identified in 18 of 22 contigs.

We investigated risk factors for fecal colonization with *mcr-1*–carrying bacteria separately for small-scale farms and household farms because a joint model did not converge due to inflated sampling weight assigned to household chicken farms (online Technical Appendix 1 Table 1). Multivariate analysis identified the presence of younger chickens (<20.5 weeks old) and the use of

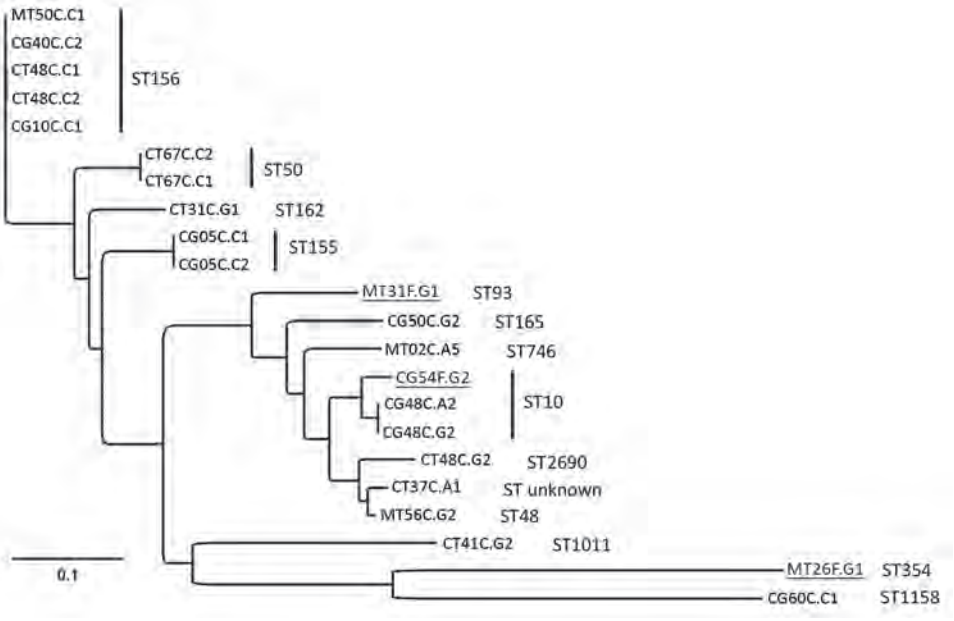

Figure. Phylogenetic analyses of *mcr-1*–positive *Escherichia coli* isolated from chickens and chicken farmers, Vietnam, 2012–2013. Maximum-likelihood tree of 22 *mcr-1*–carrying *E. coli* isolated from 15 chicken fecal samples and 3 human fecal swab samples (underlined), constructed by using CSI Phylogeny 1.4 (https://cge.cbs.dtu.dk//services/CSIPhylogeny/), shows a genome-wide single-nucleotide polymorphism (SNP) comparison. A total of 74,585 SNPs were concatenated for pairwise comparison (difference between pairs 0–32,267 SNPs). The multilocus sequence types (ST) are indicated next to the isolate names. The ST155 isolates CG05C.C1 and CG05C.C2 differ by 1 SNP; the ST10 isolates CG48C.A2 and CG48C.G2 differ by 1 SNP and 1 antimicrobial resistance gene; the ST156 isolates CT48C.C1 and CT48C.C2 differ by 4 SNPs and 3 antimicrobial resistance genes; and the ST50 isolates CT67C.C1 and CT67C.C2 are phenotypically different but have 0 SNP differences and originate from the same sample and are therefore likely to be highly related or identical. Scale bar indicates number of nucleotide substitutions per site.

Table 2. Multivariate analysis of risk factors associated with fecal colonization with *mcr-1*–carrying bacteria in small-scale chicken farms (N = 94) and in humans (N = 440), Vietnam, 2012–2013*

Variables	No. tested	No. *mcr-1*–positive	OR (95% CI)	p value
Small-scale chicken farms				
Age of chickens				
Chickens <20.5 weeks old	47	32	21.3 (5.8–78.5)	<0.001
Chickens ≥20.5 weeks old	47	8	Referent	
Use of colistin	21	14	5.1 (1.4–18.8)	0.017
Humans				
Urban persons†	88	8	Referent	
Rural persons†	173	31	2.1 (0.9–5.0)	0.075
Farmers exposed to *mcr-1*–negative chickens	91	16	1.8 (0.7–4.7)	0.205
Farmers exposed to *mcr-1*–positive chickens	88	29	5.3 (2.2–12.7)	<0.001

*OR, odds ratio.
†Not involved in poultry farming.

colistin as independent risk factors for fecal colonization with *mcr-1*–carrying bacteria in chickens (odds ratios [ORs] 21.3 and 5.1, respectively) in small-scale farms (Table 2). We were unable to identify potential risk factors associated with fecal colonization with *mcr-1*-carrying bacteria in chickens in household farms. Among human participants, farmers who were exposed to *mcr-1*–positive chickens showed a significantly increased risk for colonization with *mcr-1*–carrying bacteria (OR 5.3; Table 2) in contrast with urban individuals not involved in chicken farming, rural individuals not exposed to chickens, and farmers with *mcr-1*–negative chickens.

Conclusions

Our study shows that colonization with *mcr-1*–carrying bacteria in chickens is associated with colistin usage and colonization of humans is associated with exposure to *mcr-1*–positive chickens. These findings suggest that colistin use is the main driver for the observed high prevalence (59.4%) of *mcr-1* in fecal samples from chickens, with zoonotic transmission explaining the high prevalence (34.7%) in farmers. Zoonotic transmission of colistin-resistant *E. coli* from a domesticated pig (*11*) and companion animals (*12*) to humans has been reported.

We found that younger chickens were more likely to be colonized with *mcr-1*–carrying bacteria than older chickens (≥20.5 weeks), probably because of the higher antimicrobial treatment incidence in younger chickens (74.0 [interquartile range 0–278]/1,000 chickens treated daily with 1 defined daily dose) than in older chickens (46.3 [interquartile range 0–124]/1,000 chickens treated daily with 1 defined daily dose) (N.V. Trung, unpub. data). However, our study was insufficiently powered to detect such an association in multivariate analysis. In addition, the gastrointestinal tract of younger chickens might be colonized by antimicrobial-resistant bacteria more readily than older chickens (*13*).

The spread of the *mcr-1* gene on different plasmid types (IncI2, IncHI2, and IncHI2A) might explain its successful spread in different *E. coli* clones. We also identified the IS*Apl1* transposon in 81.8% (18/22) of our isolates. Because this genetic element is involved in horizontal gene transfer, it is likely to be a key factor contributing to the widespread dissemination of *mcr-1* (*14*).

Our study is subject to several limitations. First, the cross-sectional study design precludes the demonstration of direct transmission of the *mcr-1* gene between chickens and humans. Second, the presence of colistin in chicken feeds could not be verified and thus misclassification of farms in terms of their colistin use was possible. Last, we did not screen for the *mcr-2* gene, which is also involved in colistin resistance (*15*).

In summary, our results show an association between colistin use on farms and the presence of the *mcr-1* gene in animals. Given the potentially serious consequences of the spread of the *mcr-1* gene from food production animals to humans, prudent use of antimicrobial drugs in animal production should be enforced globally, including in small-scale and household farms.

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Disseminated Nontuberculous Mycobacteria in HIV-Infected Patients, Oregon, USA, 2007–2012

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We determined disseminated nontuberculous mycobacteria incidence in the HIV-infected population of Oregon, USA, during 2007–2012 by using statewide laboratory surveillance. We identified 37 disseminated nontuberculous mycobacteria cases among 7,349 patients with median annual incidence of 110/100,000 HIV person-years and the highest incidence in those with CD4 counts <50 cells/mm^3 (5,300/100,000 person-years).

Nontuberculous mycobacteria (NTM) are ubiquitous in the environment and are increasingly implicated in human diseases worldwide. Disseminated NTM infections are seen exclusively among immunocompromised hosts, including those with AIDS (*1*–*3*). Historically, disseminated *Mycobacterium avium* complex (MAC) has been among the most common AIDS-presenting diagnoses, with a 49% case-fatality rate at 1 year (*2*,*4*). After 1987, the number of reported disseminated NTM infections grew with the prevalence of AIDS; in 1990, between 15% and 24% of US residents with AIDS had disseminated NTM (primarily MAC) (*4*–*6*).

After the US Public Health Service formally recommended prophylaxis for MAC prevention in 1993 and highly active antiretroviral therapy (HAART) became widespread in 1996, disseminated MAC in HIV patients declined. Incidence estimates of 10,000 cases/100,000 person-years in 1992 declined to 250 cases/100,000 person-years in 2007 in the United States; however, patient populations studied were limited to those established in specialty HIV care (*7*–*9*). Modern-day population-based estimates are lacking. Having access to statewide NTM isolates and HIV case reporting, we sought to generate a population-based estimate of disseminated NTM disease incidence among patients infected with HIV in Oregon, USA.

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The Study

We used statewide reported NTM isolate data from all laboratories in Oregon collected during 2007–2012. From these data, we defined disseminated NTM as a reported NTM isolate from blood, cerebrospinal fluid, bone marrow, lymph node, or other sterile site in an Oregon resident ≥18 years of age. In a sensitivity analysis, we expanded this definition to include isolates from nonsterile sites, specifically skin and urine, that could still represent disseminated disease. To identify cases of HIV-associated disseminated NTM, we linked patients with NTM isolates to the statewide HIV surveillance registry by name, date of birth, and sex using Registry Plus Link Plus (*10*). We reviewed all possible matches manually.

To calculate incidence, exposure time began with the date of HIV diagnosis. We censored cases at death, NTM diagnosis, or 1 year after the last reported CD4 or viral load result when the gap was >2 years between laboratory tests. Since 2006, Oregon law has required that laboratories report all CD4 counts and viral loads to the Oregon Health Authority. We calculated exposure time within CD4 ranges for each interval between reported CD4 counts until patients were censored. We calculated disseminated NTM incidence by CD4 count (closest to NTM diagnosis within preceding 3 months) and year with 95% CIs under the Poisson distribution in SAS version 9.4 (*11*). The Oregon Health Authority determined this project to be public health surveillance.

We identified 37 disseminated NTM cases among a population of 7,349 HIV-infected patients with 33,072 person-years of exposure; most were male (28, 75.7%), non-Hispanic (29, 78.4%), and white (26, 70.3%), with a median age of 40.0 years (range 22.7–59.4 years) (Table 1). Median time from HIV diagnosis to disseminated NTM was 2.1 years (range 0–20.7 years). Most cases were caused by MAC (35, 94.6%); 19 patients (52.8%, 1 outcome missing) died, with median survival of 0.3 years (range 0–5.8 years) after NTM diagnosis.

Median CD4 count and viral load collected closest to NTM diagnosis were 10 cells/mm^3 and 131,446 copies/mL, respectively. At the time of NTM isolation, 23 (74.2%) patients had CD4 counts <50 cells/mm^3, 5 (16.1%) had CD4 counts 50–99 cells/mm^3, 1 (3.2%) had a CD4 count 100-199 cells/mm^3, and 2 (6.5%) had CD4 counts ≥200 cells/mm^3. Of the 2 patients with CD4 counts

Table 1. Characteristics of 37 patients with HIV and disseminated NTM infection, Oregon, USA, 2007–2012*

Variable	Value
Race	
White	26 (70.3)
Black	6 (16.2)
Asian, Native American, Alaskan Native, Pacific Islander, ≥1 race	5 (13.5)
Hispanic	8 (21.6)
Female sex	9 (24.3)
Age at disseminated NTM diagnosis, y, median (range)	40 (22.7–59.4)
Deceased at time of data analysis	19 (52.8)
Time from HIV diagnosis to NTM diagnosis, y, median (range)	2.1 (0–20.7)
NTM species	
M. aubagnense	1 (2.7)
M. avium	35 (94.6)
M. chelonae	1 (2.7)
CD4 count, cells/mm^3)†	10 (1–414)
<50	23 (74.2)
50–100	5 (16.1)
101–200	1 (3.2)
>200	2 (6.5)
Viral load, copies/mL, median (range)	131,446 (0–4,570,000)

*Values are no. (%) except as indicated. NTM, nontuberculous mycobacteria.
†CD4 count missing for 6 patients.

≥200 cells/mm^3, both had previous CD4 counts ≥200 cells/mm^3; 1 had an undetectable viral load, and the other had persistent viremia >200,000 copies/mL. The patient with a CD4 count of 100-199 cells/mm^3 at the time of disseminated NTM diagnosis had a CD4 count of 32 cells/mm^3 9 months before diagnosis.

CD4 testing frequency varied substantially in the 6,171 patients with reported CD4 counts during 2007–2012. Median time between CD4 count tests for those with testing intervals <2 years was 76 days (range 0–664 days). Six patients with disseminated NTM did not have CD4 counts during the 3-month period before diagnosis and were excluded from CD4-specific incidence.

The highest incidence of disseminated NTM (5,300/100,000 person-years) occurred in patients with CD4 counts <50 cells/mm^3 (Table 2). Annual incidence ranged from 50/100,000 to 200/100,000 person-years (median 110/100,000), with no statistically significant difference over the 6 years.

An additional 10 cases of possible disseminated NTM infections were identified (4 skin, 1 wound, 1 urine, 2 abscess, 2 unknown site). Species included MAC (*7*), *Mycobacterium xenopi* (*1*), and unspeciated (*2*). All 10 cases occurred in white men of median age 45.2 years (range 32.0–62.8 years). None of these persons had a CD4 count <50 cells/mm^3. The 9 cases with CD4 test results (1 did not have a CD4 test within 3 months of the study) were distributed evenly among the other 3 CD4 count groups (median 135 cells/mm^3, range 92–352 cells/mm^3). Two of these 10 patients had died by the time of the analysis.

Conclusions

Limited studies have evaluated the incidence of disseminated NTM disease in persons with HIV in the HAART era (*7*,*9*). We evaluated the complete population of Oregon residents with HIV and NTM during 2007–2012 by merging 2 comprehensive statewide databases. Estimated annual incidence (0.11/100 person-years) was lower than estimates previously described in the HAART era (0.25–2/100 person-years) (*9*,*12*). During our study period, 32% (n = 1,996) of Oregon's HIV-infected population had ≥1 CD4 count <200 cells/mm^3 and 10.9% (n = 677) had ≥1 CD4 count <50 cells/mm^3, indicating a relatively small population at theoretic risk for disseminated NTM. Of the 677 patients at highest risk, with ≥1 CD4 count <50 cells/mm^3, disseminated NTM developed in only 3.4%.

Mortality rates from disseminated NTM remain high, with median survival and mortality rates similar to those shown in data reported previously (*4*,*6*,*7*,*12*). Three persons who had disseminated NTM had CD4 counts >99 cells/mm^3. Most studies evaluating disseminated NTM are limited to patients with CD4 counts <99 cells/mm^3; however, Nightingale et al. found that 7% of patients with a previous AIDS-defining illness and disseminated NTM disease had CD4 counts >99 cells/mm^3 (maximum 441 cells/mm^3) (*4*). Our findings are consistent with this and

Table 2. Incidence of disseminated nontuberculous mycobacterial disease, by CD4 count closest to diagnosis date, Oregon, USA, 2007–2012*

	CD4 count, cells/mm^3			
Rate	<50, n = 677	50–100, n = 784	101–200, n = 1,697	>200, n = 5,827
Incidence/100,000 person-years (95% CI)	5,300 (3,360–7,950)	950 (310–2,210)	60 (0–310)	10 (0–30)

*n values indicate no. patients with ≥1 CD4 count in specified category.

suggest that disseminated NTM should be considered in this population.

Oregon is a state with a relatively low HIV prevalence (143.1 cases/100,000 persons during 2007–2012), with a range of 71–79 HIV deaths annually and an average of 256 new cases yearly (*13*). Our data might have limited generalizability beyond Oregon, given that most patients living with HIV in Oregon have access to care; 87% of all patients in care have a suppressed viral load, and only 16% of AIDS Drug Assistance Program clients lack reported recent CD4 or viral load test results (*13*).

Analysis of incidence by CD4 count was also limited by variability in frequency of laboratory testing, which was determined by the treating clinicians. Six (16%) patients with disseminated NTM did not have CD4 counts taken 3 months before or 1 month after diagnosis. In addition, laboratory results could have been missing in Oregon residents who receive HIV care outside the state.

Our clinical data were limited to laboratory results; therefore, we were unable to analyze the impact of antiretroviral therapy, antimicrobial prophylaxis or treatment, and access to care. Given the rarity of this infection in our cohort, we suspect that prophylaxis is routinely being used in those with CD4 count <50 cells/mm^3; however, mortality rates continue to be high in those in whom disseminated NTM infections develop.

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Autochthonous Outbreak and Expansion of Canine Visceral Leishmaniasis, Uruguay

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We report an outbreak of canine visceral leishmaniasis in Uruguay. Blood specimens from 11/45 dogs tested positive for *Leishmania* spp. Specimens of *Lutzomyia longipalpis* sand flies were captured; typing revealed *Leishmania infantum*. Our findings document an expansion of visceral leishmaniasis to southern South America and risk for vectorborne transmission to humans.

Visceral leishmaniasis (VL) is a zoonotic disease caused by flagellated protozoa of the genus *Leishmania* and transmitted by sand flies belonging to the Phlebotominae subfamily; those of the *Lutzomia longipalpis* species are the main vectors. VL affects humans and canids; canids are identified as the main reservoir of the parasite (*1*). This zoonosis has been endemic in northeastern Brazil for several centuries, but it has been recently expanding to southern areas of the South American continent (*2*–*4*). In 2010, the presence of the vector *L. longipalpis* sand flies was recorded for the first time in Uruguay (*5*); the right environmental conditions, the presence of competent sand fly vectors, and the constant appearance of new cases of canine and human leishmaniasis in border countries have made Uruguay susceptible to VL transmission (*5*).

In 2015, we performed a house-by-house survey in Arenitas Blancas (31°25.000′S, 58°00.066′W) in Salto, Uruguay. We included 49 dogs in the survey. Whole-blood samples from 11 (22%) tested positive for *Leishmania* spp. with 2 different diagnostic kits, TR DPP (Bio-Manguinhos, Rio de Janeiro, Brazil) and Speed Leish K (Virbac, Carros, France), both of which detect antibodies raised against *Leishmania* antigens in whole blood, plasma, or serum by immunochromatographic methods. Among the dogs whose specimens tested positive, 8 showed the common clinical signs of skin lesions, fever, weight loss, and eye lesions; 3 were asymptomatic. Dogs whose specimens tested positive came from 9 different houses in the same neighborhood (Figure, panel A); of these, 2 dogs had never traveled outside their residence, and in 2 other cases, both dam and offspring were infected. Three dogs came from breeding kennels, and the rest were born in Arenitas Blancas.

We performed lymph node biopsies and bone marrow aspiration in dogs whose specimens tested positive; we also confirmed infection by direct observation of amastigotes in stained slide smears of aspirates. After extracting DNA from tissue samples by using the Quick-DNA Universal kit (Zymo Research, Irvine, California, USA), we performed PCR and sequencing of the ribosomal internal transcribed spacer 1 (*6*) to achieve typing of *Leishmania* spp. at the species level. We aligned and analyzed the sequences by using MAFFT software (*7*); the neighbor-joining phylogenetic tree obtained from the analysis showed that sequences identified from our samples group together with sequences belonging to *L. infantum* reference strains that we sequenced, as well as with sequences obtained from GenBank (Figure, panel B). Accession numbers and percentage of identity of the sequences obtained from GenBank are *L. infantum*, KM677146.1 and KC477100.1 (100%); *L. donovani*, HM130608.1 and HQ830358.1 (99%); *L. amazonensis*, DQ182536.1 (86%); *L. guyanensis*, DQ182541.1 (81%); and *L. braziliensis*, DQ182537.1 (81%).

To verify that the complete domestic cycle of *Leishmania* spp. was taking place in the affected area, we placed CDC Miniature Light Traps (John W. Hock Company, Gainesville, FL, USA) in domiciles in which affected dogs had been found. All sampling was peridomestic and consisted of 13 traps placed overnight on 3 different nights; sampling resulted in collection of 3 sand flies, 1 male and 2 female. Using observational analysis, we identified the collected samples as *L. longipalpis*; this result was confirmed by PCR with species-specific primer LiCac (*8*,*9*). Furthermore, we performed PCR amplification with *Leishmania*-specific primers AJS1 and DeB8 (*8*) using sand fly DNA as a template. A PCR product of 300 bp from one of the sand flies was amplified and sequenced and showed *Leishmania* DNA in the vector (data not shown).

In summary, we describe an autochthonous outbreak of canine VL in Uruguay. The reported cases represent the expansion of VL to southern areas of the continent; the evidence shows that *L. infantum* is the parasite responsible for the outbreak in both canine hosts and a sand fly vector. The presence of competent vectors in the area constitutes a risk for the human population. Further work is needed to implement effective measures to control the extension of cases. It is also mandatory to improve surveillance of the vector and expand surveillance to other wild and domestic potential hosts. Finally, efforts should be made to prevent new cases of human VL in Uruguay.

[1]These authors contributed equally to this article.

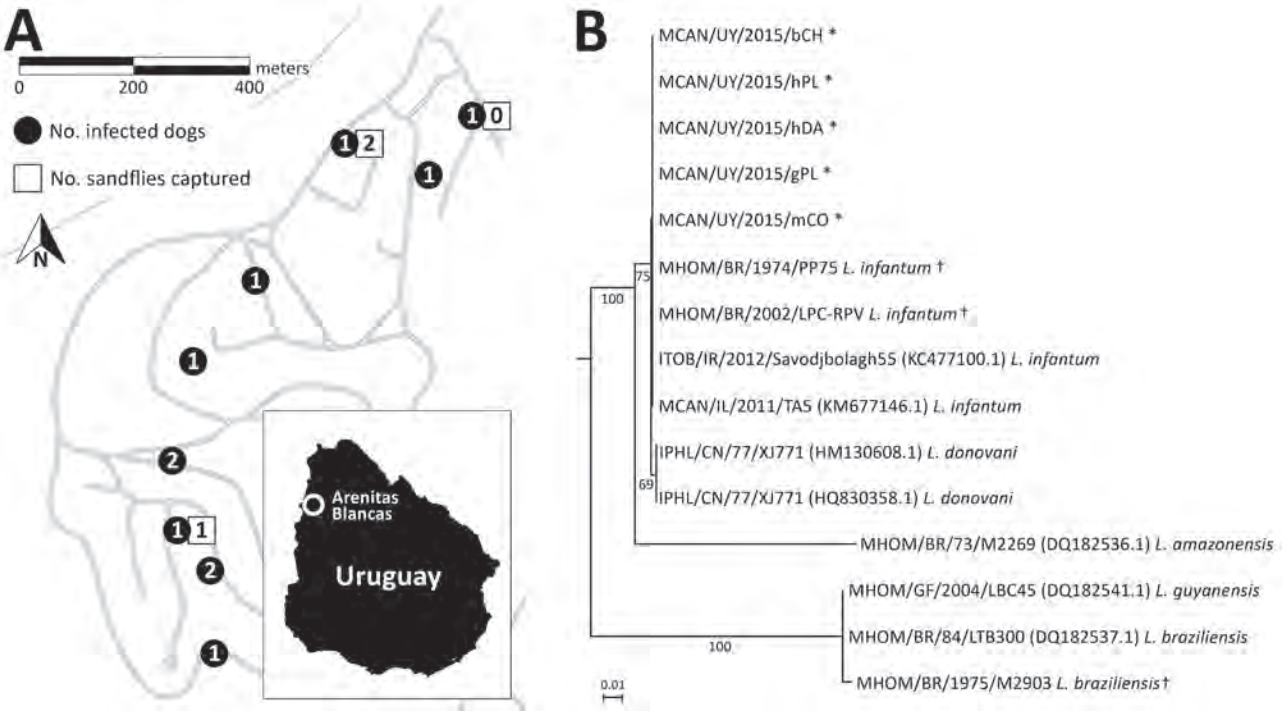

Figure. Survey of *Leishmania* spp. infection in dogs in Arenitas Blancas, Salto, Uruguay. A) Surveyed area in the locality of Arenitas Blancas in Salto, Uruguay. White squares represent the location of *Lutzomia longipalpis* sand fly captures, and black circles represent domiciles in which infected dogs were found; numbers indicate number of *Leishmania* spp.–infected sand flies or dogs at that location. B) Neighbor-joining phylogenetic tree obtained from the analysis of *Leishmania* internal transcribed spacer 1 sequences from tissue samples of infected dogs. Bootstrap values are represented at the nodes of major branches. Scale bar indicates nucleotide substitutions per site. *Sequences obtained from infected dog samples. †Reference strains sequenced by the authors.

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Worldwide Endemicity of a Multidrug-Resistant *Staphylococcus capitis* Clone Involved in Neonatal Sepsis

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A multidrug-resistant *Staphylococcus capitis* clone, NRCS-A, has been isolated from neonatal intensive care units in 17 countries throughout the world. *S. capitis* NRCS-A prevalence is high in some neonatal intensive care units in France. These data highlight the worldwide endemicity and epidemiologic relevance of this multidrug-resistant, coagulase-negative staphylococci clone.

Preterm birth is the world's leading cause of death before 5 years of age (*1*). Neonatal sepsis, mostly due to coagulase-negative staphylococci, occurs frequently in neonatal intensive care units, especially in very low birthweight preterm infants (*2*). Cases and series of neonatal sepsis involving *Staphylococcus capitis* have been reported in different countries (*3*) and were initially considered unrelated epidemic bursts. More recently, we detected a single multidrug-resistant clone of *S. capitis*, designated as the NRCS-A clone and characterized by a specific pulsed-field gel electrophoresis (PFGE) pattern, in several neonatal intensive care units (NICUs) in France, Belgium, the United Kingdom, and Australia (*4*,*5*). The clonality of the strains was confirmed by PFGE, multilocus sequence typing–like analysis, and whole-genome sequencing. We also showed that all NRCS-A isolates exhibited a decreased susceptibility to all of the antimicrobial agents frequently used in NICUs, namely β-lactams, aminoglycosides, and vancomycin (*5*). Furthermore, a recent study showed that *S. capitis* NRCS-A–associated sepsis constitutes an independent risk factor for severe illness in neonates (*6*).

We suspected that the initial report of NRCS-A dissemination in NICUs from 4 distant countries was only the tip of the iceberg and that the spread of NRCS-A strains was much wider than expected. To determine the extent of NRCS-A dissemination, we asked microbiologic laboratories worldwide to send us methicillin-resistant *S. capitis* strains isolated from blood cultures of neonates. These isolates were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and subjected to PFGE using the *Sma*I restriction enzyme as previously described (*7*). NRCS-A's characteristic PFGE pattern was found for 154 strains isolated between 1994 and 2015 in 34 NICUs from 17 countries: Australia, Belgium, Brazil, Canada, Czech Republic, Denmark, Finland, France, Germany, the Netherlands, New Zealand, Norway, South Korea, Switzerland, Taiwan, the United Kingdom, and the United States.

Retrospective, laboratory-based epidemiologic investigations to estimate the prevalence of NRCS-A strains in NICUs could not be performed on the same worldwide scale, so we conducted such a study in France. Results collected from 47 of the 57 NICUs in France during 2014 indicated that only 4 NICUs were free of NRCS-A. In the 43 other NICUs, NRCS-A strains accounted for up to 46% of all cases of positive cultures of blood from neonates (median 13%, interquartile range 10%–20%) and represented 19% of all coagulase-negative staphylococci strains isolated from the blood cultures of neonates.

Taken together, these data unquestionably demonstrate the unusual worldwide endemicity of the multidrug-resistant NRCS-A clone in NICUs. In addition, the epidemiologic data from France highlight the propensity of NRCS-A to invade and settle in most NICUs on a national scale. Once endemic in a NICU, NRCS-A strains expose infected neonates to a risk of therapeutic failure because treatment of neonatal sepsis involving methicillin-resistant coagulase-negative staphylococci is usually based on vancomycin and aminoglycosides, to which NRCS-A isolates are not susceptible (*3*–*5*).

A thorough investigation of the determinants of the worldwide spread of NRCS-A is urgently needed to unravel the dissemination routes and reservoirs of this multidrug-resistant clone and to succeed in managing and controlling its diffusion. The risk of vancomycin treatment failure warrants an investigation of alternate antimicrobial stewardship strategies, in particular linezolid, daptomycin, and ceftarolin, to treat NRCS-A–associated neonatal sepsis.

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Potentially Zoonotic *Bartonella* in Bats from France and Spain

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We detected *Bartonella* in 11 of 109 insectivorous bats from France and 1 of 26 bats from Spain. These genetic variants are closely related to bat-associated *Bartonella* described in Finland and the United Kingdom and to *B. mayotimonensis*, the agent of a human endocarditis case in the United States.

Bartonellae have been identified in bats sampled in locations around the world where diverse chiropteran host species can interact with numerous *Bartonella* variants and potential arthropod vectors (*1*–*3*). Many *Bartonella* species are zoonotic, potentially affecting human and bat health (*4*). *Bartonella* spp. in bat populations of Europe are of particular interest because some variants described in Finland and the United Kingdom are closely related to *Bartonella mayotimonensis*, a species detected in the resected aortic valve of a 59-year-old endocarditis patient in the United States (*5*,*6*). To determine if potentially zoonotic bat-associated bartonellae are circulating elsewhere in Europe, we tested insectivorous bats from France and Spain for the presence of *Bartonella* spp.

We performed necropsies on 26 bats from Spain and 109 from France to collect heart tissue for *Bartonella* spp. diagnostics (online Technical Appendix Table 1, https://wwwnc.cdc.gov/EID/article/23/3/16-0934-Techapp1.pdf). Bats from Spain were originally collected during active surveillance for rabies at the Unidad de Aislamiento y Detección Virus, Instituto de Salud Carlos III, Madrid, Spain. Of the bats from France, 97 were originally submitted for passive rabies surveillance to the Agence Nationale de Sécurité Sanitaire

de l'Alimentation,de l'Environnement et du Travail (ANSES), Laboratoire de la Rage et de la Faune Sauvage de Nancy in Malzéville, France. We sampled the remaining 12 animals from a rehabilitation center at the Musée d'Histoire Naturelle in Bourges, France. All bats collected for *Bartonella* diagnostics tested negative for rabies. Spatial coordinates were recorded for all bats at the point of sampling before submission for centralized laboratory testing (online Technical Appendix Figure). Whenever possible, we identified bat species and sex. Methods were approved by the University of California, Davis (Davis, CA, USA), Institutional Animal Care and Use Committee (protocol 17669).

We used sampling records to determine the genus and species for 118 of the 135 bats; identification data were not available for 17 of 26 bats from Spain. The 118 identified bats belonged to 8 genera and at least 13 different species: 70 *Pipistrellus* spp. (31 *P. pipistrellus*, 24 *P. nathusii*, 6 *P. kuhlii*, 4 *P. pigmaeus,* and 5 *Pipistrellus* spp.); 15 *Nyctalus noctula*; 7 *Eptesicus serotinus*; 11 *Myotis* spp. (4 *M. mystacinus*, 3 *M. daubentonii,* 1 *M. bechsteinii,* 1 *M. myotis,* 1 *M. nattereri,* 1 *M emarginatus*); 6 *Plecotus* spp. (4 *P. austriacus* and 2 *P. auritus*); 6 *Tadarida teniotis*; 2 *Barbastella barbastellus*; and 1 *Vespertilio murinus*.

We used NucleoSpin Blood QuickPure kits (Machery-Nagel, Düren, Germany) to extract DNA from 25 mg of macerated heart tissue according to the manufacturer's instructions. We used tissue spiked with *B. henselae* as a positive control for DNA extraction. We screened samples by PCR targeting the citrate synthase gene (*gltA*). Primers CSH1f (GCGAATGAAGCGTGCCTAAA) and BhCS1137.n (AATGCAAAAAGAACAGTAAACA) amplified an ≈350-bp fragment suitable for distinguishing *Bartonella* species (*7*). PCR thermal cycler parameters were set at 10 min at 95°C, followed by 40 cycles of 30 s at 94°C, 1 min at 57°C, 2 min at 72°C, 5 mins at 75°C, and infinite hold at 4°C. We verified amplicon sizes by gel electrophoresis, and Service de Séquençage de Eurofins (Paris, France) generated sequence data from PCR products.

We used OpenEpi version 3.01 (http://www.openepi.com/) to calculate descriptive statistics and CIs for prevalence data. We constructed phylogenetic trees by using the MrBayes plugin in Geneious version 8.1.7 with a Markov Chain Monte Carlo value of 1,100,000 and 100,000 burn-in length (*8*). We used the ggplot2 package in R (https://www.r-project.org/) to create spatial maps.

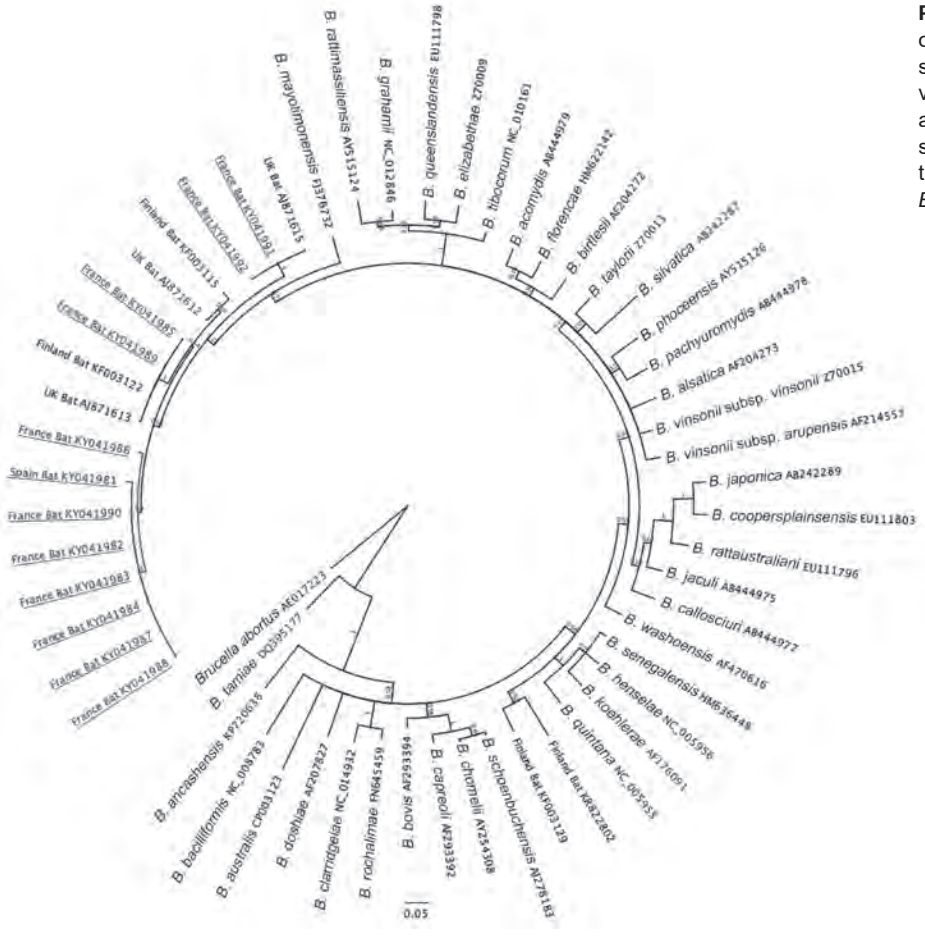

Figure. Phylogenetic analysis of citrate synthase (*gltA*) gene sequences of 12 *Bartonella* spp. variants detected in bats from France and Spain (underlined) compared with sequences from GenBank. All 12 of these variants clustered with zoonotic *B. mayotimonensis.*

We detected *Bartonella* DNA in 12 (8.9%) of 135 bat heart tissue samples (online Technical Appendix Table 2); 11 of the tissues were from bats from France, and 1 was from an unidentified bat captured in Torreferrusa, Catalonia, Spain. The 11 *Bartonella*-positive bats from France belonged to only 4 of the 13 sampled species: *N. noctula* (2/15 bats [13.3%, 95% CI 1.7%–40.5%]), *P. nathusii* (6/24 bats [25%, 95% CI 9.8%–46.7%]), *M. daubentonii* (2/3 bats [66.6%, 95% CI 9.4%–99.1%]), and *M. mystacinus* (1/4 bats [25%, 95% CI 0.6%–80.6%]).

All 12 *Bartonella* variants (GenBank accession nos. KY041981–KY041992) clustered closely with zoonotic *B. mayotimonensis* (Figure). Two sequences obtained from *M. daubentonii* bats sampled in Lorraine (GenBank accession no. KY041985) and Upper Normandy (GenBank accession no. KY041989), France, shared 100% nt identity with *Bartonella* strains previously isolated from bats of the same species in Finland and the United Kingdom (*5*,*9*). None of the *Bartonella* variants were closely related to *Candidatus* Bartonella naantaliensis or *Candidatus* Bartonella hemsundetiensis, which were also described in bats sampled in Finland (*5*,*10*). The absence of variants resembling these bartonellae from northern Europe suggests a spatial heterogeneity in the distribution of *Bartonella* spp. across bat populations and selective adaptations to specific host reservoirs.

Further research is needed to better evaluate the prevalence of zoonotic *Bartonella* species in western Europe and to determine if *B. mayotimonensis*, the agent of a US case of human endocarditis, is present across a broader range than currently documented. Future studies should consider specifically focusing on *Nyctalus, Pipistrellus,* and *Myotis* bat species, from which we most frequently detected variants similar to *B. mayotimonensis*.

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Two Cases of *Neisseria meningitidis* Proctitis in HIV-Positive Men Who Have Sex with Men

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We report 2 cases from Spain of infectious proctitis caused by *Neisseria meningitidis* in HIV-positive men who have sex with men. Genetic characterization of the isolates showed that they are unusual strains not found in other more frequent meningococcal locations. This finding suggests an association between specific strains and anogenital tract colonization.

Pathogens that cause proctitis include *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Treponema pallidum*, and herpes simplex virus (*1*). We report 2 cases from Spain of proctitis caused by *Neisseria meningitidis*, a pathogen less frequently detected.

The first case-patient was a 32-year-old HIV-positive man who had proctalgia and purulent anal and urethral discharges. He reported having unprotected sex with other men.

The second case-patient was a 49-year HIV-positive man who had a purulent discharge, pain, and anal tenesmus. He reported having unprotected anal sex with other men and having previously diagnosed sexually transmitted infections.

Both patients were given a diagnosis of probable infectious proctitis. Rectal exudates samples were collected for detection of infectious agents. The first case-patient was given ceftriaxone (1 g, single intramuscular dose) and azithromycin (1.5 g, single oral dose). The second case-patient was given ceftriaxone (250 mg, single intramuscular dose). Both patients showed clinical improvement.

Routine screening for *Mycoplasma* spp. and nucleic acid amplification for *N. gonorrhoeae* and *C. trachomatis* yielded negative results. We isolated gram-negative diplococci from both patients on modified Martin-Lewis agar (Becton Dickinson, Franklin Lakes, NJ, USA) and identified these diplococci as *N. meningitidis* serogroup B by using mass spectrometry (Biotyper System; Bruker, Billerica, MA, USA) and standard biochemical tests (Vitek II; bioMérieux, Marcy l'Etoile, France).

We determined MICs by using Etest (bioMérieux). MICs were 0.047 mg/L for the isolate from first patient and 0.19 mg/L for the isolate from the second patient for tetracycline; 0.125 and 0.016 mg/L for cefotaxime; 0.004 and 0.006 mg/L for ciprofloxacin, 0.75 and 2 mg/L for azithromycin; 0.047 and 0.25 mg/L for penicillin; 0.38 and 1.5 mg/L for ampicillin; <0.002 and <0.002 mg/L for ceftriaxone; and 0.064 and 0.094 mg/L for rifampin.

The isolates were sent to the National Reference Laboratory in Madrid, Spain, for confirmation of identification and characterization. Molecular characterization included genotyping by sequencing variable regions of the PorA protein gene, (*2*), multilocus sequence typing (MLST) (*3*), and FetA protein variable region gene characterization (*4*).

The isolates were identified by using slide agglutination with specific polyclonal antibodies as being *N. meningitidis* serogroup B. The isolate from first case-patient was characterized as genosubtype P1.22,14-13 (PorA VR1:22, VR2:14-13), FetA type F5–7 (FetA VR:5-7), sequence type (ST) 10866, with a clonal complex (CC) not assigned (NA) (i.e., a B:P1.22,14-13:F5-7:ST10866 CCNA strain). The isolate from second case-patient was characterized as B:P1.17-6,23-6:F3-36:ST3469 (CC4821).

We did not find similar isolates at the National Reference Laboratory for patients with invasive meningococcal disease (IMD), healthy carriers, or persons with urogenital infections. Only 4 strains in the same CC as that for the isolate from the second case-patient were found in the MLST database (http://pubmlst.org/neisseria/http://pubmlst.org/neisseria/); these isolates had a similar genosubtype and FetA type, but only 1 isolate, obtained from a carrier in Australia in 2014, had the same ST.

B:22,14 strains are found more frequently in IMD patients and healthy carriers. However, more strains are ST-213CC, which is the second most prevalent CC in Spain. We found 2 strains with the same ST as the strain from the first case-patient in the MLST database. Both of these strains were isolated from men who had sex with men in Brighton, UK, 1 isolated in 2013 from a urethral swab specimen (22-4,14-13:F5-7:ST10866) and the other isolated in 2012 from rectal swab specimen (22,14-13:F5-7:ST10866).

The natural habitat of *N. meningitidis* is the human nasopharynx. However, it occasionally enters the bloodstream and causes IMD characterized by meningitis or septicemia (*5*). *N. meningitidis* has been isolated from the urethra, cervix, and anal canal and has been reported as a cause of anogenital infection (*6*–*8*). Orogenital contact is the most probable route of *N. meningitidis* transmission from nasopharynx to urogenital tract and anal canal (*8*), which has been associated mainly with heterosexual patients (*8*). The pathogenic role for rectal infection with *N. meningitidis*

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is unclear because of a low frequency of symptomatic infected patients. However, histopathologic changes have been reported in rectal mucous of patients infected with *N. meningitidis* (*9*).

N. meningitidis is highly variable because it can naturally undergo transformation, which leads to changes in virulence and transmissibility and suggests that new variants could emerge that have increased fitness for alternative/novel niches (*10*). This suggestion could be useful in identifying *N. meningitidis* strains with ST10866, which have been isolated from patients with anogenital infections and might be one of those variants. Whether HIV infection, with its associated immune problems, favors colonization with other microorganisms adapted to different ecologic niches has not been resolved.

Although an increased prevalence of meningococcal anogenital infections has been reported (*6*–*8*), the incidence of these infections is probably still underestimated because *N. meningitidis* might be the etiologic agent in patients with gonococcal-like urethritis and proctitis. This underestimation could be caused, in part, by use of PCR as the only diagnostic method. Thus, culture is still needed for isolating strains and determining their antimicrobial drug resistance. Monitoring the incidence of meningococcus reproductive tract infections and genetic characterization are necessary to determine the magnitude and clinical role of these infections.

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Association of Diabetes and Tuberculosis Disease among US-Bound Adult Refugees, 2009–2014

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Diabetes is associated with an increased risk for active tuberculosis (TB) disease. We conducted a case–control study and found a significant association between diabetes and TB disease among US-bound refugees. These findings underscore the value of collaborative management of both diseases.

The burden of tuberculosis (TB) is highest in resource-limited countries, many of which are experiencing increased rates of diabetes (*1*,*2*). Because of the effect of diabetes on the immune system, risk for active TB disease

is higher and treatment outcomes are poorer among persons with diabetes (*3*). In 2011, the World Health Organization and the International Union Against Tuberculosis and Lung Disease developed a framework for a coordinated response to both diseases and advised screening all TB patients for diabetes (*4*).

Annually, ≈70,000 refugees resettle in the United States (*5*). Before departure, all refugees undergo a medical examination and screening for conditions of public health importance; TB is considered a priority condition (*6*). The Centers for Disease Control and Prevention Electronic Disease Notification (EDN) System captures data from these examinations (*7*). Domestic screening examinations are recommended within 3 months of arrival in the United States, and the TB component is captured in EDN.

Using EDN, we examined the association of diabetes and TB disease in our target population, which consisted of 249,037 US-bound refugees ≥18 years of age at the time of their overseas medical examination who arrived in the United States from January 1, 2009, through August 31, 2014. We excluded 187 records because of missing data. TB disease was defined as clinical or laboratory-diagnosed disease, either 1) active pulmonary or extrapulmonary TB diagnosed during the overseas examination prior to departure and treated before arrival in the United States (*6*) or 2) diagnosis of active TB at the domestic examination after entry into the Unites States.

Diabetes screening is not a requirement for admission to the United States. However, if reported by the refugee while recording the medical history or discovered during the overseas examination process, diabetes should be documented on the medical examination forms. Using text parsing techniques described previously (*8*), we searched for evidence of diabetes in these forms.

Demographic variables were sex, age group, living setting (refugee camp or noncamp setting), and region of nationality, which were assigned according to US Department of State categories. Body mass index was categorized as underweight (<18.5 kg/m^2), normal (18.5 to <25 kg/m^2), overweight (25 to <30 kg/m^2), or obese (≥30 kg/m^2).

We used logistic regression to assess the association of diabetes and TB and assessed effect modification between region and diabetes. Variables were included in the multivariate model if they were significant ($p<0.05$) in a model of diabetes only or if they were confounders, defined as variables causing a change in odds between diabetes and TB of >20%.

From January 1, 2009, through August 31, 2014, according to our case definitions, 2,262 (0.9%) of 248,850 US-bound refugees ≥18 years of age had TB, 5,767 (2.3%) had diabetes, and 56 (<0.1%) had both. Effect modification between region and diabetes was not significant. After controlling for region, sex, age group, body mass index, and living in a refugee camp, we found a significant association between diabetes and TB (adjusted odds ratio 1.7, 95% CI 1.3–2.2) (Table).

Although the link between diabetes and TB is widely accepted, previous studies showed differing strengths of association and significance (*4*), which could be attributed to variability in the prevalence of diabetes and TB in the population. We found a modest association between diabetes and TB disease.

Table. Characteristics of US-bound adult refugees and association of diabetes with TB disease, Electronic Disease Notification System, January 2009–August 2014*

Characteristic	Total no. (%), N = 248,850	TB, no. (%), n = 2,262	Odds ratio (95% CI)	
			Univariate	Multivariate
Diabetes	5,767 (2.3)	56 (2.5)	1.1 (0.8–1.4)	1.7 (1.3–2.2)
Region				
Africa	40,731 (16.4)	422 (18.7)	Reference	Reference
East Asia and the Pacific	58,701 (23.6)	971 (42.9)	1.6 (1.4–1.8)	1.7 (1.5–1.9)
Europe and Eurasia	3,867 (1.6)	22 (1.0)	0.5 (0.4–0.8)	0.6 (0.4–1.0)
Near East	76,752 (30.8)	44 (2.0)	0.1 (<0.1–0.1)	0.1 (0.1–0.1)
South and Central Asia	52,677 (21.2)	802 (35.5)	1.5 (1.3–1.7)	1.2 (1.0–1.4)
Western Hemisphere	16,122 (6.5)	1 (<0.1)	<0.1 (<0.1–<0.1)	<0.1 (<0.1–<0.1)
Female sex	115,297 (46.3)	747 (33.0)	0.6 (0.5–0.6)	0.6 (0.5–0.6)
Age group, y				
18–44	190,141 (76.4)	1,391 (61.5)	Reference	Reference
45–64	45,711 (18.4)	600 (26.5)	1.8 (1.6–2.0)	2.6 (2.3–2.8)
65–74	9,397 (3.8)	175 (7.7)	2.6 (2.2–3.0)	3.6 (3.0–4.3)
≥75	3,601 (1.5)	96 (4.2)	3.7 (3.0–4.6)	5.0 (4.0–6.3)
BMI category†				
Underweight	20,300 (8.6)	390 (18.3)	1.7 (1.5–1.9)	1.6 (1.4–1.8)
Normal	127,033 (53.7)	1,465 (68.8)	Reference	Reference
Overweight	58,299 (24.7)	233 (10.9)	0.3 (0.3–0.4)	0.5 (0.5–0.6)
Obese	30,731 (13.0)	43 (2.0)	0.1 (0.1–0.2)	0.4 (0.3–0.5)
Lived in refugee camp‡	88,490 (36.0)	1,448 (64.6)	3.3 (3.0–3.6)	1.2 (1.0–1.3)

*Adults were those ≥18 years of age. BMI, body mass index; TB, tuberculosis.
†Proportions based on nonmissing data; 5.0% missing data.
‡Proportions based on nonmissing data; 1.2% missing data.

This evaluation was subject to limitations. We were not able to control for all risk factors for TB (e.g., HIV), which could have affected our odds calculations. Also, because diabetes screening is not a required part of the overseas medical examination, some persons with diabetes were probably missed, leading to an underestimation of the true prevalence of diabetes in this population. In the United States, ≈28% of persons have undiagnosed diabetes (*9*); this number may be greater among refugees with limited access to healthcare services (*10*). Because diabetes was significantly associated with TB, a differential misclassification may have occurred where there was more undiagnosed diabetes among refugees with a history of TB disease. If misclassification of diabetes status did occur, these findings are an underestimation of the actual strength of association between diabetes and TB. More research, such as testing for diabetes during overseas medical examinations would allow for a more accurate assessment.

Most state refugee health programs rescreen all refugees for TB as well as other infectious diseases (e.g., hepatitis B) at the time of arrival in the United States. Some states also test for diabetes. Our findings, along with the extensive literature associating diabetes with TB, indicate that a diagnosis of TB disease in a patient should trigger testing for diabetes to optimize treatment. In states that already screen for both diseases, further research could lead to promising innovation in collaboratively managing the 2 diseases.

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Molecular Verification of New World *Mansonella perstans* Parasitemias

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We obtained ribosomal and mitochondrial DNA sequences from residents of Amazonas state, Brazil, with *Mansonella* parasitemias. Phylogenetic analysis of these sequences confirm that *M. ozzardi* and *M. perstans* parasites occur in sympatry and reveal the close relationship between *M. perstans* in Africa and Brazil, providing insights into the parasite's New World origins.

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Mansonella perstans is one of the most prevalent and poorly understood parasites known to cause parasitemias in humans (*1*–*3*). An estimated 114 million persons are infected with *M. perstans* parasites in Africa alone, and *M. perstans* parasitemias have also been repeatedly reported to occur in continental South America (*1*,*2*). In Uganda, *M. perstans* infections and parasitic loads have been shown to map closely with the larval breeding sites of its known vector, the *Culicoides* midge (*1*). Almost nothing is known about the parasites' epidemiology in continental South America; however, it has been established that simuliids and a diverse range of Ceratopogonid vector species transmit *M. ozzardi* parasites in Latin America (*1*). Thus, it cannot safely assumed that the epidemiology of *M. perstans* in Latin America is particularly similar to its epidemiology in Africa (*1*,*2*).

Like most reports of *M. perstans* in Africa, reports of the occurrence of *M. perstans* in South America have almost always been based on morphologically identified microfilariae observed in blood smears (*1*,*2*). However, in contrast to the situation in Africa, where only 1 parasitemia-causing *Mansonella* parasite occurs, reports of *M. perstans* in South America have been limited to equatorial rainforest regions, where other *Mansonella* parasitemia-causing parasites also commonly occur (*1*–*4*). Therefore, microscopy-based *Mansonella* parasitemia diagnoses in Latin America can be regarded as more prone to error than those made in Africa (*1*–*6*). Conspicuously, *M. perstans* DNA sequences originating outside of Africa have until now been missing, and the relationship between *M. perstans* in Africa and *M. perstans* in the New World has been a mystery (*1*).

By using 3 DNA sequences commonly used in the molecular systematics of filarial parasites (the nuclear internal transcribed spacer 1 [ITS1]–based ribosomal DNA sequence [*7*] and the mitochondrial 12S and cytochrome c oxidase subunit 1 genes [*6*]), we confirmed *M. perstans* microfilariae morphologic identifications made using thick blood smears prepared from persons residing in the village of São Gabriel da Cachoeira, Amazonas state, Brazil. Besides providing verification of *M. perstans* morphologic identifications, the ITS1 sequences generated for this study

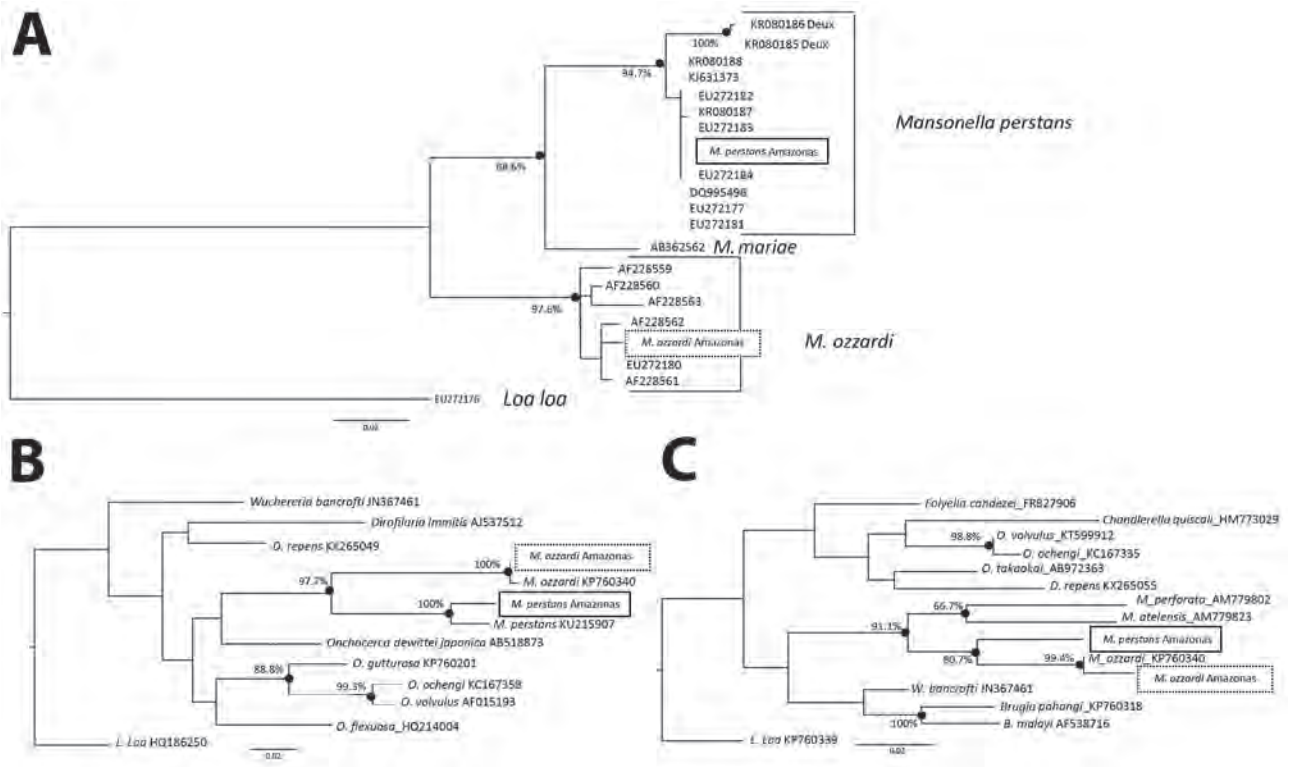

Figure. Maximum-likelihood phylogenetic trees showing the relationship between *Mansonella* parasites from Amazon region of Brazil (Amazonas state) and some of their closest relatives. A) Ribosomal internal transcribed spacer 1–based phylogeny. B) Mitochondrial cytochrome c oxidase subunit 1–based phylogeny. C) Mitochondrial 12S-based phylogeny. All 3 trees were prepared by using DNA sequence alignments and PHYLIP version 3.67 (http://evolution.genetics.washington.edu/phylip.html). Black circles indicate significant bootstrap-supported nodes as a percentage of 1,000 pseudoreplicates. Solid boxes indicate *M. perstans* and dashed boxes *M. ozzardi* sequences generated for this study and used in the construction of the displayed trees. Scale bars indicate nucleotide substitutions per site. These sequence have been submitted to GenBank and EMBL (accession nos.: *M. perstans* cytochrome c oxidase subunit 1, LT623909; *M. ozzardi* cytochrome c oxidase subunit 1, LT623910; *M. perstans* 12S, LT623913; *M. ozzardi* 12S, LT623914; *M. perstans* internal transcribed spacer 1, LT623911; and *M. ozzardi* internal transcribed spacer 1, LT623912).

allowed a phylogenetic analysis with *M. perstans* from Africa. The ribosomal ITS1 *M. perstans* from Brazil clustered with other *M. perstans* ITS1 sequences originating from Africa in a strongly (>94%) bootstrap-supported *M. perstans*–exclusive monophyletic group (Figure). Similarly, *M. ozzardi* ITS1 sequences obtained from parasites from Brazil clustered in another strongly (>97%) bootstrap-supported monophyletic group containing only *M. ozzardi* origin sequences.

The genetic distance between the ITS1 sequences of *M. perstan*s from Brazil and their closest relatives from Africa is very small (corresponding to <1% divergence across 396 nucleotide positions). From the ITS1-based phylogenetic analysis, the *M. perstans* from Brazil appear to be more closely related to some *M. perstans* in Africa than they are to others. The ITS1 sequences from *M. perstans* previously described as *M. perstans* "deux" (*8*) and originating from Gabon can be observed in a bootstrap-supported cluster forming a sister clade to the bootstrap-supported monophyletic cluster containing the *M. perstans* from Brazil, which also contains sequences originating from Cameroon, Côte d'Ivoire, Equatorial Guinea, Gabon, Mali, and Sierra Leone. Thus, our results suggest that *M. perstans* arrived in Latin America after the standard form of *M. perstans* diverged from the *M. perstans* "deux" form.

Sequences from mitochondrial genes 12S rDNA and cytochrome c oxidase subunit 1 have also been recovered from blood samples in Brazil and used to confirm morphologic and ITS1-based *Mansonella* parasite identifications (*6*). Phylogenetic analysis performed with these mitochondrial gene segments was consistent with our ITS1 analysis and also suggest that *M. perstans* arrived in Latin America very recently (Figure1). In addition to verifying that South America does indeed have the conditions to support *M. perstans* and providing a useful reference for vector incrimination and other epidemiologic studies, our findings have also provided insights into the origin of the *M. perstans* parasite in South America. Given how similar our findings are to those obtained when *Onchocerca volvulus* parasite mitogenomes from Latin America and Africa have been compared, they suggest that *M. perstans*, like *O. volvulus,* probably arrived in Latin America as a consequence of the slave trade (*9*–*10*).

The work presented in this study was performed as part of a study called "Mansonelose em área urbana de São Gabriel da Cachoeira, Amazonas," which received ethical clearance from the Comité de Ética em Pesquisa do Instituto Oswaldo Cruz (CAAE: 41678515.1.0000.5248) and financial support from the Fundação de Amparo à Ciência e Pesquisa of Amazonas state (processo 062.00647/2014) and Programa Pesquisa Sistema Único de Saúde and Programa de Excelência em Pesquisa Básica e Aplicada em Saúde project grants (processo 062.02005/2014) awarded by the Fundação de Amparo à Pesquisa do Estado do Amazonas.

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Imported *Leptospira licerasiae* Infection in Traveler Returning to Japan from Brazil

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We describe a case of intermediate leptospirosis resulting from *Leptospira licerasiae* infection in a traveler returning to Japan from Brazil. Intermediate leptospirosis should be included in the differential diagnosis for travelers with fever returning from South America. This case highlights the need for strategies that detect pathogenic and intermediate *Leptospira* species.

Leptospirosis, caused by spirochetes of the genus *Leptospira*, is a neglected zoonotic disease found in tropical and subtropical regions. *Leptospira* species are classified into 3 groups on the basis of 16S rRNA gene sequences: pathogenic, intermediate, and saprophytic groups. Although *Leptospira* species from the pathogenic group are considered to be the main cause of leptospirosis, Chiriboga et al. reported that most cases of leptospirosis in Ecuador were caused by intermediate species (*1*). We describe a case of leptospirosis caused by *L. licerasiae*, an intermediate species, in a traveler returning to Japan from Brazil.

In late November 2015, a previously healthy 40-year-old Japanese man sought treatment at the National Center for Global Health and Medicine (Tokyo, Japan) with a high fever and shaking chills. He had recently spent 15 days in Corumbá, Brazil, where he worked as part of a camera crew in mid-November 2015. He used insect repellent during his trip but had been bitten by mosquitoes many times while walking and swimming in waist-deep water in the Brazilian wetlands. His symptoms began 6 days after his return from Brazil and included high fever, chills, arthralgia and myalgia in his elbow and knee joints, and burning skin pain over the his whole body for the 24 hours before he sought treatment. At the time of his first visit to this clinic, he reported new onset retroorbital pain and shaking chills.

On examination, his body temperature was 39.5°C and his pulse rate was relatively low (87 beats/min). He had mild congested bulbar conjunctivae, localized urticaria on his trunk, and many small, old injury scars on both of his legs (online Technical Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/22/3/16-1262-Techapp1.pdf). Results of rapid antigen detection tests and Giemsa stains of blood smears for *Plasmodium* spp. were negative for 3 consecutive days. Results of laboratory tests were negative, including IgM, IgG, and NS-1 antigen tests against dengue virus; HIV screening; rapid antigen detection test against influenza virus; and blood and urine cultures. Moreover, PCR results for *Leptospira* and for dengue, chikungunya, and Zika viruses were negative.

Treatment with ceftriaxone (2 g 1×/d) was initiated 1 day after hospital admission. Four hours after infusion began, the patient's fever rose to 40°C, which was considered a Jarisch-Herxheimer reaction. Fever resolved the next day. Laboratory test results showed elevated total bilirubin (2.3 mg/dL [reference range 0.3–1.2mg/dL]), aspartate aminotransferase (62 U/L [13–33 U/L]), alanine aminotransferase (73 U/L [8–42 U/L]), lactic acid dehydrogenase (456 U/L [119–229]), and C-reactive protein (13.6 mg/dL [0–0.3 mg/dL]), but these values quickly returned to within reference ranges. Three days after ceftriaxone treatment began, all symptoms had resolved, and the patient was discharged from the hospital with a prescription for doxycycline (100 mg 2×/d).

At the time of discharge, 3 days after the blood culture was set up, spirochetes were observed in Korthof and EMJH media. Nucleotide sequencing of the 16S rRNA gene of the isolate, NIID18 (Japan National Bioresource of Bacterial Pathogens no. 18467, http://pathogenic.lab.nig.ac.jp/)

Table. Case descriptions of patients infected with *Leptospira licerasiae* in South America*

Patient no.	Age, y/sex	Location	Occupation	Symptoms	Therapy	Prognosis	Ref.
VAR10	31/F	Varillal, Peru	Food vendor	2-d history of fever, malaise, chills, headache, dizziness	Antipyretics	Resolved 5 d later	(*7*)
HAI029	19/F	Iquitos, Peru	Student/ domestic worker	5-d history of fever, malaise, chills, headache, dizziness, leg pain and weakness, abdominal pain, anorexia, nausea, vomiting	None	Resolved†	(*7*)
NIID18	40/M	Corumbá, Brazil	Camera crew	1-d history of fever, shaking chills, retroorbital pain, arthralgia and myalgia in elbow and knee joints, burning skin pain over the whole body, congested bulbar conjunctivae	CTRX 2 g/d for 4 d, then DOX 100 mg 2×/d for 3 d	Resolved within 3 d after initiation of CTRX	This study

*CTRX, ceftriaxone; DOX, doxycycline; ref., reference.
†The day when the symptoms resolved was not described in the report.

(online Technical Appendix Figure 2), revealed it to be *L. licerasiae*: the sequence (GenBank accession no. LC164227) had 99.3% identity (1,339/1,348 bp) with VAR 010 (GenBank accession no. EF612284), the type strain of *L. licerasiae*. The partial *flaB* sequence of the NIID18 isolate (GenBank accession no. LC164228) also showed the highest similarity with VAR 010 (96.6%, GenBank accession no. LC005426). NIID18 did not react with a panel of antisera for 18 serovars (*2*). An increase in antibody titers in paired serum samples was observed against the isolate (reciprocal titers 50 and 200 in acute- and convalescent-phase samples, respectively), according to microscopic agglutination test (*3*). After receiving antimicrobial drug therapy for 7 days, the patient had completely recovered.

The intermediate *Leptospira* group comprises 5 species: *L. licerasiae, L. wolffii, L. fainei, L. broomii,* and *L. inadai*. Although this species group has been detected in environmental soil and water samples from the Southeast Asia (*4*–*6*), human cases involving returned travelers have not been well-documented previously (*1*,*7*–*10*). To our knowledge, only 2 cases of *L. licerasiae* isolation from a human host have been reported; such isolations were first reported in Peru in 2008 (7) (Table), although many serum samples from febrile patients in the Peruvian Amazon have reacted with an *L. licerasiae* isolate. Members of *Rattus* species are considered major reservoir hosts (*7*).

We were unable to detect *Leptospira* DNA in the case-patient's blood using *flaB*-nested PCR because this method is specific to species in the pathogenic group. The patient received a diagnosis of leptospirosis after *L. licerasiae* was isolated from a blood culture. Therefore, PCR targeting conserved genes among genus *Leptospira*, such as 16S rRNA, is more suitable not only for clinical situations but also for epidemiologic studies.

This case highlights the need for including leptospirosis caused by intermediate group species in the differential diagnosis for patients with fever who have recently returned from South America. In addition, we emphasize the utility of genes such as 16S rRNA for detecting pathogenic and intermediate *Leptospira* groups.

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Serologic Evidence for MERS-CoV Infection in Dromedary Camels, Punjab, Pakistan, 2012–2015

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Dromedary camels from Africa and Arabia are an established source for zoonotic Middle East respiratory syndrome coronavirus (MERS-CoV) infection among humans. In Pakistan, we found specific neutralizing antibodies in samples from 39.5% of 565 dromedaries, documenting significant expansion of the enzootic range of MERS-CoV to Asia.

The Middle East respiratory syndrome coronavirus (MERS-CoV) is a zoonotic pathogen that causes severe respiratory disease in humans. Dromedary camels (Camelus dromedarius), which have 1 hump on their backs, have been identified as an animal reservoir and source of human MERS-CoV infection (*1*). Reports document widespread infection of these camels on the Arabian Peninsula and parts of Africa (*2*–*4*). Besides these regions, dromedaries are also native to several countries in Asia. A study in Kazakhstan found no evidence for MERS-CoV infection in dromedaries (*5*). Absence of MERS-CoV infection in dromedary camels in Asia could mean a vulnerable animal reservoir at risk for de novo introduction by sporadic contact (e.g., by trade) with dromedaries from MERS-CoV endemic areas. Pakistan is 1 of 8 countries globally, and the only country outside Africa, that has a dromedary population exceeding 1 million animals (FAOSTAT database; http://faostat3.fao.org). Because of the limited capacities for routine MERS-CoV surveillance and a considerable human population size in countries in northeastern Asia, targeted investigation of the MERS-CoV infection status is of interest for global public health agencies.

In this study, we examined dromedaries from Pakistan for exposure to MERS-CoV. We tested 565 serum samples, which we collected from 348 female and 217 male animals by using a convenience sampling strategy in 9 districts of Punjab, eastern Pakistan, during 2012–2015. The median age of the animals was 5 years.

The testing algorithm comprised 2 antibody detection methods (*3*,*6*). MERS-CoV IgG was detected by using a MERS-CoV camel antibody ELISA (EUROIMMUN, Lübeck, Germany). We tested all serum samples that exceeded a cutoff of 0.4, validated in previous studies (*3*,*6*), by using a microneutralization (MN) test for confirmation (*3*). Only serum samples with neutralizing activity ≥1:80 were considered MERS-CoV antibody–positive.

A total of 315 (55.8%) of 565 camel serum samples exceeded the ELISA signal cutoff (Table). Of these, 223 (39.5%) were confirmed by using MN. We identified MERS-CoV neutralizing antibodies in camels sampled in all study years and in nearly all regions except the district of Chiniot (Table). The rate of neutralizing antibody–positive camel samples ranged from 82.9% in Rahim Yar Khan to 24.1% in the Jhang district (Table).

By using a merged dataset comprising all regions, we correlated seropositivity to animal age and sex. Sex-dependent differences suggested pronounced seropositivity in male animals, but differences were not significant ($p>0.067$; online Technical Appendix Table, https://wwwnc.cdc.gov/EID/article/23/3/16-1285-Techapp1.pdf). Seropositivity increased with age: samples from more than half (51.1%) of all animals >5 years of age and less than one third (29.2%) of animals <2 years of age tested positive ($p<0.001$; online Technical Appendix Table). These age-dependent differences are similar to those found in previous studies (*6*,*7*) and can be explained by long-lasting immune response or regular reexposure after initial MERS-CoV infection. The finding of antibodies in young camels born in Pakistan suggests ongoing circulation of MERS-CoV in the country.

Bactrian camels (*C. bactrianus*), which have 2 humps on their backs, are also native to Central and East Asia. Studies of these camels in China and Mongolia, as well as dromedary and Bactrian camels in southern Kazakhstan, uniformly reported absence of MERS-CoV during 2014–2015 (*5*,*8*,*9*). However, dromedaries may become sources of infection for Bactrian camels that are susceptible to MERS-CoV and present in this vast geographic range. The possibility of cross-species transmission within the order of camelids has been documented by a study that found signs of MERS-CoV infection in alpacas (*Vicugna pacos*) that shared a barn with dromedaries in Qatar (*10*). The absence of MERS-CoV in camelid populations in northeastern parts of Asia (Mongolia,

[1]These authors contributed equally to this article.

Table. Detection of Middle East respiratory syndrome coronavirus antibodies in dromedary camels from different districts of Punjab Province, Pakistan, 2012–2015*

District	Year of sampling†	No. animals tested	ELISA		MN	
			No. positive	Detection rate, % (95% CI)	No. positive	Detection rate, % (95% CI)
Chiniot	2012, 2014	23	4	17.4 (5–38.8)	0	0 (0.0–14.8)
Faisalabad	2012, 2013, 2015	66	45	68.2 (55.6–79.1)	32	48.5 (36.0–61.1)
Jhang	2012, 2013, 2015	220	79	35.9 (29.6–42.6)	53	24.1 (18.6–30.3)
Bhakkar	2012	88	51	57.9 (47–68.4)	36	40.9 (30.5–51.9)
Layyah	2012	13	9	69.2 (38.6–90.9)	4	30.8 (9.1–61.4)
Muzaffargarh	2012, 2013	40	26	65 (48.3–79.4)	15	37.5 (22.7–54.2)
Bahawalpur	2015	29	26	89.7 (72.6–97.8)	21	72.4 (52.8–87.3)
Lodhran	2014	51	42	82.4 (69.1–91.6)	33	64.7 (50.1–77.6)
Rahim Yar Khan	2012	35	33	94.3 (80.8–99.3)	29	82.9 (66.4–93.4)
Total		565	315	55.8 (51.5–59.9)	223	39.5 (35.4–43.6)

*Serum samples were tested at a dilution of 1:100; MNT was done in a microtiter plate format in duplicate at a dilution of 1:80. ELISA ratio >0.4 and MN >1:80 were considered positive. MN, microneutralization.
†Samples from all years, except the samples from 2012 and 2014 from Chiniot, were MN positive.

Kazakhstan, and China) is compatible with the view that the spread of MERS-CoV from Africa into Asia may be a recent development. However, other explanations, including resistance to infection by Bactrian camels, are possible. Studies of susceptibility should be conducted to clarify whether Bactrian camels in Asia could act as a naïve reservoir population in the future.

Based on MERS-CoV antibodies in dromedary camels, our data suggest a risk for human exposure in Punjab, Pakistan, that is similar to risks in Africa and the Arabian Peninsula. Of note, Punjab shares a border with the state of Rajasthan in India, which harbors that country's largest dromedary population. A similar risk for human exposure is likely for this part of India. However, findings of antibodies against MERS-CoV in migrant workers from these areas should be interpreted with caution because these workers often employed in Arabian countries. For Pakistan, our data largely exclude the scenario of a widely susceptible animal reservoir population in which de novo introduction of MERS-CoV could start an epizootic that could lead to spillover epidemics among humans.

Acknowledgments

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Fungal Contamination of Methylprednisolone Causing Recurrent Lumbosacral Intradural Abscess

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Fungal meningitis transmitted through injections of methylprednisolone contaminated with Exserohilum rostratum affected 753 persons and caused 61 deaths in the United States in 2012. We report a case of infection recurrence after 24 months with manifestation of an intradural fungal abscess. Recurrent fungal disease should remain on the differential diagnosis list for previously exposed patients.

Fungal meningitis transmitted through injections of methylprednisolone contaminated with *Exserohilum rostratum* affected 753 persons and caused 61 deaths in the United States in 2012 (*1*). More than 13,400 patients were potentially exposed to 1 of 3 contaminated drug lots (*2*,*3*). However, whether recommended treatment eliminates this disease is unknown because of limited reports of recurrent disease (*4*).

As of December 2015, the Centers for Disease Control and Prevention (CDC) had reported 8 cases of *E. rostratum* meningitis relapses within a median time of 90 days. Reporting recurrent cases informs potential treatment recommendation changes and long-term care guidelines for affected persons. With institutional review board approval from Wake Forest University Medical Center, we describe fungal infection recurrence at 24 months.

A 78-year-old woman sought treatment at the neurosurgery clinic at Wake Forest Medical Center (Winston-Salem, NC, USA), in August 2015 with a 4-month history of acute or chronic lower back pain, leg weakness, and radicular pain in the left side. Nonoperative interventions, including narcotics, physical therapy, and epidural steroid injections (L5–S1, July 2015), did not control her symptoms.

The patient had received an injection of contaminated methylprednisolone on September 12, 2012. She was identified as a patient affected by the contamination and contacted as part of the CDC investigation. One month after contact, she was hospitalized for intractable headaches, nausea, and vomiting.). PCR results from 2 samples of cerebrospinal fluid (CSF) performed by CDC were positive for *E. rostratum* (from October 16 and 22, 2012). Treatment consisted of intravenous voriconazole, later switched to ambisome, with a transition to oral voriconazole due to hallucinations. Therapy response was monitored with serial lumbar punctures. Contrast-enhanced magnetic resonance imaging (MRI) of the lumbar region was performed on November 18, 2012, and showed an enhancing epidural abscess, spanning T12–S2. Treatment was discontinued on January 16, 2013, a decision supported by 10 serially negative CSF fungal cultures and repeat PCR, negative for *E. rostratum,* performed at CDC on February 22, 2013. Lumbar MRI on February 1, 2013, showed improvement of the lumbar epidural abscess.

The October 2012 hospitalization was complicated by the patient's persistent altered mental status and right hemiparesis, which prompted a contrast-enhanced MRI of the brain on February 15, 2013, that demonstrated a left transverse sinus thrombus. The condition was monitored, and repeat imaging on March 1, 2013, found it to be nearly resolved. The patient appeared to be recovered from her infection at her last infectious disease follow-up in March 2013.

The patient was hospitalized again in May 2015 after a fall; she experienced worsening back pain, headaches, and confusion. Brain MRI demonstrated a recurrent dural venous thrombosis, which was treated with anticoagulants. Given concern for recurrent fungal infection, she also underwent lumbar puncture. CSF cell count and chemistries were within normal limits. CSF cultures were negative for fungi.

At home, her acute left leg pain worsened, leaving her nonambulatory, and she sought hospital management. On neurologic examination, she was awake and alert with some delirium/confusion and some mild weakness in the left lower leg. Contrast-enhanced lumbar MRI demonstrated a homogeneous enhancing intradural mass, spanning L4 to the sacrum, with a corresponding T2 hypointense signal (online Technical Appendix Figure, panels A, B, https://wwwnc.cdc.gov/EID/article/23/3/16-1334-Techapp1.pdf). Diagnosing this lumbosacral intradural mass was not obvious because the differential diagnosis includes neoplasms, infections, and hematomas. Given the patient's worsening symptoms, we performed nerve decompression and resection of the mass.

During the operation, the patient underwent a laminectomy of L3–L5; intraoperative findings showed an intradural abscess and arachnoiditis, with edema and adherence of the cauda equina nerve roots (Figure). Pathologic examination demonstrated abundant necrotic material containing septate hyphae fungal elements of brown pigment, consistent with a dematiaceous fungus. The material did not undergo PCR, given her clinical history (including PCR) and pathologic findings at recurrence. Her

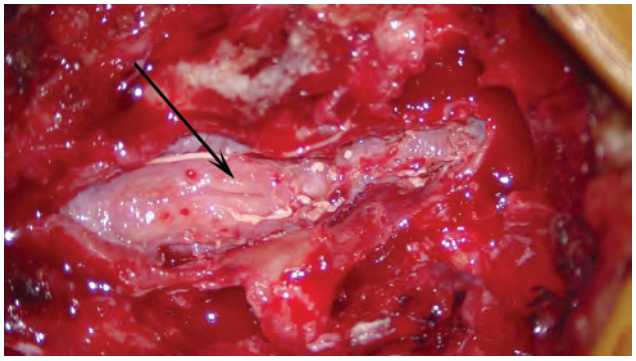

Figure. Intraoperative image demonstrating postevacuation cauda equina nerve roots that are grossly edematous and adherent (arrow), consistent with arachnoiditis, in a patient with recurrent infection from fungal-contaminated methylprednisolone, North Carolina, USA, 2015.

condition was treated with intravenous amphotericin B and voriconazole during her 12-day hospitalization, and she was discharged on oral voriconazole for outpatient treatment, with an anticipated duration of 1 year. At 5-month follow-up, she had complete resolution of her back pain and was full strength with some intermittent left radicular pain.

Only 3 other cases of intradural abscess were reported from the initial outbreak, making this recurrence a notable CNS disease manifestation (*5*). The patient had several risk factors for recurrence. She had received epidural steroid injections after antifungal treatment; the steroids resulted in an immunocompromised environment, potentially allowing for immune evasion and residual disease. A dural rent during multiple spinal taps or posttreatment epidural steroid injections may have seeded the fungus in the intradural space, which then expanded because antifungal agents demonstrate relatively poor CSF penetration. She also underwent a 3-month initial treatment; at least 6 months of antifungal treatment is currently recommended, although optimal treatment duration remains uncertain because objective criteria for infection clearance are lacking.

Given the potential for recurrence, fungal disease should remain on the differential diagnosis list for patients with prior exposure. In addition, long-term follow-up could identify patients needing further treatment (*4*).

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Successful Treatment of Human Plague with Oral Ciprofloxacin

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The US Food and Drug Administration recently approved ciprofloxacin for treatment of plague (*Yersina pestis* infection) based on animal studies. Published evidence of efficacy in humans is sparse. We report 5 cases of culture-confirmed human plague treated successfully with oral ciprofloxacin, including 1 case of pneumonic plague.

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Plague is a life-threatening zoonotic disease caused by *Yersinia pestis*. Zoonotic foci exist on several continents; however, resource-poor areas in sub-Saharan Africa account for most human cases (*1*). The pathogenesis of plague involves facultative intracellular infection of host macrophages, followed by fulminant extracellular growth and bacteremia (*2*). In the absence of effective antimicrobial drug treatment, bubonic plague is fatal in ≈50% of cases and pneumonic plague in >90% (*1*,*3*).

Drugs approved by the US Food and Drug Administration (FDA) for treatment of plague include streptomycin and doxycycline. Streptomycin is bactericidal but rarely used because of limited availability and serious toxicities. Doxycycline is bacteriostatic and lacks concentration-dependent activity or a postantibiotic effect, which might limit its efficacy for serious *Y. pestis* infections (*4*). Nevertheless, low cost and oral dosing have made doxycycline a first-line treatment in several countries (*5*,*6*). Fluoroquinolones, include ciprofloxacin, have recently been approved by the FDA for treatment of plague based on animal and in vitro studies (*4*,*7*,*8*). Clinical experience with these agents, however, is limited (*1*,*5*).

During 2011–2014, patients with suspected plague seen at 6 clinics and 2 hospitals in the West Nile region of Uganda were offered enrollment in an open-label study evaluating the safety and efficacy of ciprofloxacin for treatment of plague. Patients were excluded if they were pregnant, <8 years of age, considered too ill to receive oral treatment, or had received antimicrobial drug treatment in the preceding 7 days. After written consent was obtained, diagnostic samples were collected and oral ciprofloxacin administered for 10 days at a weight-calibrated dosage of ≈15 mg/kg twice daily (range 13–17 mg/kg), with a maximum dose for adults of 750 mg twice daily. Diagnostic samples were cultured on sheep blood agar and suspect isolates confirmed by bacteriophage lysis (*9*). Patients were monitored daily during treatment, and clinical outcome was assessed 14–21 days after initial evaluation. Because of simultaneous prevention efforts and lower than expected enrollment, the study was terminated early. The study was approved by Institutional Review Boards at the Uganda Virus Research Institute, the Uganda National Council for Science and Technology, and the US Centers for Disease Control and Prevention.

Five patients with culture-confirmed plague were enrolled and treated with oral ciprofloxacin (Table). Median patient age was 27 years (range 10–52 years); median time between illness onset and enrollment was 4 days (range 1–7 days). Four patients had bubonic plague, with *Y. pestis* isolated from bubo aspirates or blood cultures. The fifth patient, a 13-year-old boy, had pneumonic plague as indicated by hemoptysis, patchy bilateral infiltrates on chest radiograph, and *Y. pestis* isolated from sputum. The illness had evolved over 6 days, a clinical course suggestive of secondary rather than primary pneumonic plague (*3*); the primary focus of infection was not identified.

Three patients were admitted and 2 treated as outpatients. In addition to ciprofloxacin, all received acetaminophen, and 2 received a bolus of normal saline. All became afebrile within 2 days. At 14 days, all had been discharged and returned to their normal activities. The 13-year-old boy with culture-confirmed pneumonic plague reported mild, nonproductive cough, but no complications were identified.

Fluoroquinolones have pharmacokinetic properties that make them attractive for treatment of plague, including bactericidal activity, good oral bioavailability, excellent tissue penetration, and an established safety record (*8*,*10*). In vitro assays suggest that ciprofloxacin is comparable to streptomycin and superior to doxycycline or gentamicin for killing of intracellular *Y. pestis* (*4*), and efficacy has been demonstrated in rodent and nonhuman primate models (*8*). Along with FDA approval, our results add to growing clinical experience (*5*) and support the broader use of oral ciprofloxacin for treatment of human plague, especially in resource-poor areas where intravenous treatment is limited.

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Table. Demographic and clinical characteristics of 5 patients with culture-confirmed plague (*Yersina pestis* infection) who were treated successfully with oral ciprofloxacin, Uganda, 2011–2014

Patient no.	Age, y/sex	Length of illness, d*	Symptoms	Laboratory evidence	Ciprofloxacin dose, mg†
1	10/F	7	Fever, left axillary bubo	Bubo, blood cultures positive	250
2	52/F	4	Fever, right axillary bubo	Bubo, blood cultures positive	650
3	27/F	1	Fever, left inguinal bubo	Bubo, blood cultures positive	750
4	36/M	1	Fever, left axillary bubo	Blood culture positive	625
5	13/M	6	Fever, chest pain, cough, blood-tinged sputum	Sputum culture positive, blood culture negative	375

*At time treatment was sought.
†Orally, twice daily; ≈15 mg/kg bodyweight with a maximum of 750 mg.

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Mycobacterium tuberculosis Infection in Free-Roaming Wild Asian Elephant

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Postmortem examination of a wild Asian elephant at Rajiv Gandhi National Park, India, revealed nodular lesions, granulomas with central caseation, and acid-fast bacilli in the lungs. PCR and nucleotide sequencing confirmed the presence of *Mycobacterium tuberculosis*. This study indicates that wild elephants can harbor *M. tuberculosis* that can become fatal.

Tuberculosis (TB), a pandemic, highly contagious disease caused by *Mycobacterium tuberculosis* complex, has affected up to one third of the world's human population. The South-East Asia Region (SEAR), which contains nearly one fourth of the world population, alone accounts for 38% of illnesses and 39% deaths caused by TB worldwide. India accounts for 58% of all forms of TB in SEAR and 55.6% of deaths caused by TB (excluding those among HIV-positive persons) in SEAR (*1*).

M. bovis is widespread in domestic animals and has been extensively documented in both captive and free-ranging wildlife. Although *M. tuberculosis* is primarily a pathogen of humans (*2*), it has been reported in zoo species (*3*,*4*) as well as in a formerly captive elephant in Africa (*5*) and a free-roaming elephant in Sri Lanka (*6*). We report the pathology and molecular characterization of *M. tuberculosis* in a wild Asian elephant (*Elephas maximus*) that had no known history of human contact and present implications for wildlife health.

In February 2016, a carcass of an ≈65-year-old free-roaming wild Asian elephant was found in the forest of Rajiv Gandhi National Park (RGNP), Karnataka, India. On postmortem examination, the lungs showed widely disseminated white-yellowish firm nodules with central caseous necrosis, distributed throughout the parenchyma (Figure, panel A). The bronchial and mediastinal lymph nodes were enlarged with nodular areas of caseous necrosis and calcification. Impression smears from the cut surfaces of lungs on staining by Ziehl-Neelsen method showed bundles of pink-stained acid-fast organisms.

DNA extracted from the lung tissue were subjected to PCR targeting amplification of a conserved region on *M. tuberculosis* complex by using forward primer 5′-GACCACGACCGAAGAATCCGCTG-3′ and reverse primer 5′-CGGACAGGCCGAGTTTGGTCATC-3′ (*7*), which yielded a specific amplicon of 445 bp, indicating presence of a pathogenic mycobacterium. To detect *M. bovis*, we used forward primer 5′-CACCCCGATGATCTTCTGTT-3′ and reverse primer 5′-GCCAGTTTGCATTGCTATT-3′ to amplify an 823-bp region on a 12.7-kb fragment of *M. bovis*. To detect *M. tuberculosis*, we used forward primer

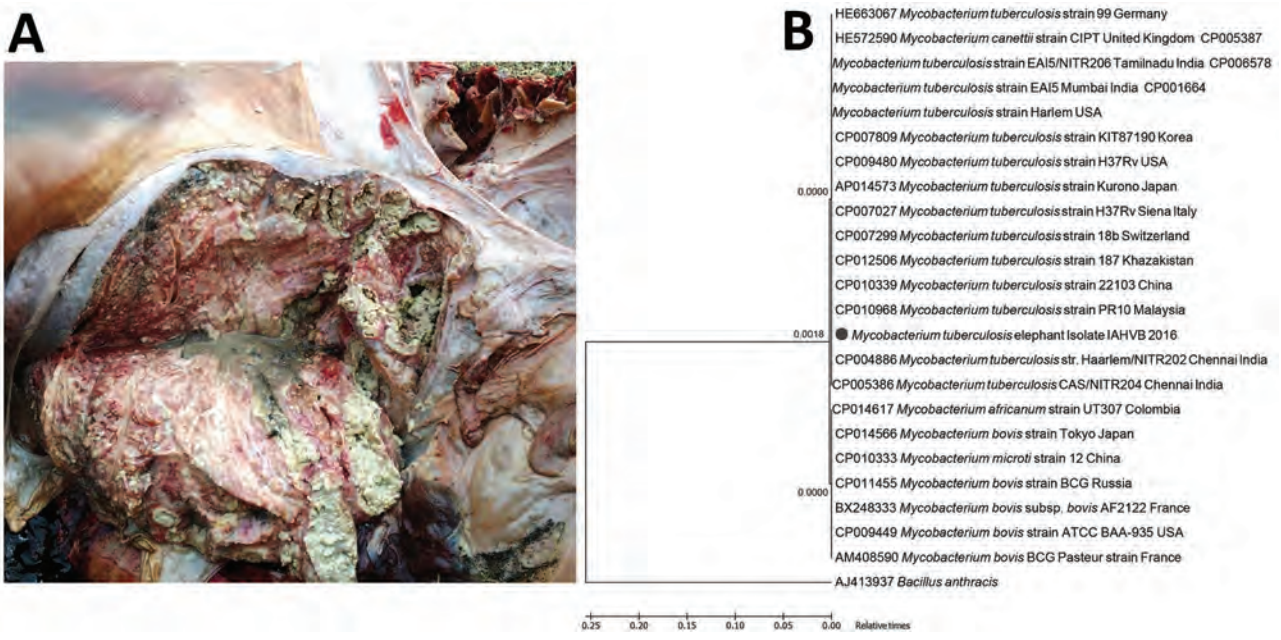

Figure. Findings from a deceased wild free-roaming Asian elephant (*Elephas maximus*) infected with *Mycobacterium tuberculosis*, Rajiv Gandhi National Park, Karnataka, India, 2016. A) Results of postmortem examination of the lungs. Note the widely disseminated firm nodules with central caseous necrosis. B) Phylogenetic analysis.

5′-CACCCCGATGATCTTCTGTT-3′ and reverse primer 5′-GACCCGCTGATCAAAGGTAT-3′ to amplify a 389-bp region on a 12.7-kb fragment of *M. tuberculosis* (*7*). The PCR used to detect *M. bovis* did not yield amplifications. PCR used to detect *M. tuberculosis* yielded a specific amplicon of 389 bp, indicating the presence of *M. tuberculosis* in the lung tissue.

Nucleotide sequencing of the obtained 389-bp amplicon and subsequent phylogenetic analysis using MEGA6 software (*8*) showed 100% nt sequence identity with *M. tuberculosis* isolates deposited in GenBank (Figure, panel B), confirming the pathogen as *M. tuberculosis*. The distance map indicated that the isolate was of Indian origin. Lung samples processed and subjected for histopathologic examination in accordance with standard protocols (*9*) showed multiple granulomas, each with central caseum, surrounded by inflammatory cells and a fibrous capsule.

The gross pathology, histopathology, PCR detection, and phylogenetic analysis confirmed the infection as TB caused by the human pathogen *M. tuberculosis*. However, the uniqueness of this investigation is that this elephant had no known history of contact with humans, making the source of infection difficult to determine. Because ecologic, environmental, and demographic factors influence the emergence of disease (*10*), the infection in this elephant could be attributable to one of the following reasons. Although RGNP is an uninhabited forest, eco-tourist activities give tourists limited access to wildlife areas. Furthermore, a highway connecting 2 states, Karnataka and Kerala, passes through RGNP, enabling transit of large numbers of human through the forest. A possibility also exists that wild elephants could accidentally enter villages adjoining the forest areas in search of feed and water.

Although remote possibilities, these events can create opportunities for susceptible wildlife populations to be exposed to human pathogens. If the elephant was not infected by accidental human contacts, then it must have acquired the disease in the wild, which leads to a larger question: can wildlife species maintain and spread *M. tuberculosis* to other susceptible species in the wild?. Comprehensive studies are needed to assess the status of TB among wild animals and to examine whether wildlife can be a potential reservoir of the disease. Irrespective of source of the infection, our study indicates that elephants living in the wild can harbor *M. tuberculosis*, which can become clinical and fatal.

Dr. Chandranaik is a veterinarian working as a scientist at the Research and Disease Investigation Division of the Institute of Animal Health and Veterinary Biologicals. His research interests include the molecular epidemiology of zoonotic pathogens.

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Mycobacterium bovis in a Free-Ranging Black Rhinoceros, Kruger National Park, South Africa, 2016

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In 2016, an emaciated black rhinoceros (*Diceros bicornis*) was found in Kruger National Park, South Africa. An interferon-γ response was detected against mycobacterial antigens, and lung tissue was positive for *Mycobacterium bovis*. This case highlights the risk that tuberculosis presents to rhinoceros in *M. bovis*–endemic areas.

Black rhinoceros (*Diceros bicornis*) are under severe threat from poaching and habitat loss. This species has been designated as critically endangered by the International Union for Conservation of Nature Red List (*1*). An estimated population of 5,000–5,445 animals are found in southern and eastern Africa, with just over 1,200 of those in South Africa (*2*). In Kruger National Park (KNP) in South Africa, the black rhinoceros population size is estimated at 400. KNP is considered an endemic area for *Mycobacterium bovis*, with cases reported in at least 12 wildlife species, including African buffalo, lion, kudu, and warthog (*3*).

Sporadic cases of tuberculosis (TB) caused by *M. tuberculosis* or *M. bovis* have been reported in black rhinoceros housed in zoos or under semi-intensive management (*4*). Although *M. bovis* is present in livestock and other wildlife species in countries in Africa where rhinoceros populations are currently present, no cases of TB have been reported in free-ranging black rhinoceros.

On June 17, 2016, rangers in KNP reported a weak, emaciated, adult female black rhinoceros that had been stationary for 36 hours in the southern area of the park (25°7′16″S, 31°55′2″E). The discovery of this animal might have resulted from increased surveillance related to poaching. When veterinary staff arrived, the rhinoceros was unresponsive and recumbent and lifted its head only when darted. External injuries were not obvious. Because of its poor prognosis, the animal was euthanized after being immobilized. Postmortem evaluation revealed an emaciated animal (body condition score 1 out of 5, http://www.daff.qld.gov.au/__data/assets/pdf_file/0015/53520/Animal-HD-Investigation-Condition-scores.pdf) with a subjectively heavy ectoparasite load. The subcutaneous and internal fat stores were reduced, consistent with the poor general body condition. Although teeth were worn, they appeared sufficient for mastication, and well-chewed ingesta was found in the gastrointestinal system. No grossly abnormal changes were found in the organs examined, except for the lungs and lymph nodes. On palpation of the lungs, numerous firm, focal, and irregular masses, 1–6 cm in diameter, were present in the right and left dorso-cranial two thirds of the lung lobes, with symmetric lesion distribution. On cut section, most lesions had a fibrous capsule and contained creamy necro-caseous material. Impression smears from the lung lesions revealed numerous acid-fast bacilli.

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The heparinized whole blood samples that were collected before the animal was euthanized were incubated in Nil and TB Antigen tubes of the QuantiFERON TB Gold In-Tube system (QIAGEN, Venlo, Netherlands) and with pokeweed mitogen (Sigma-Aldrich Pty., Ltd., Johannesburg, South Africa) as a positive control. After 24 h, plasma was harvested and interferon-γ (IFN-γ) was measured in these samples by using a bovine IFN-γ ELISA (Mabtech AB, Nacka Strand, Sweden) as previously described for African buffaloes (*5*). IFN-γ concentrations measured in the sample from the Nil tube, pokeweed mitogen tube, and TB Antigen tube were 4 pg/mL, 753 pg/mL, and 175 pg/mL, respectively. The TB antigen-specific release of IFN-γ was consistent with immunologic sensitization to *M. bovis* or *M. tuberculosis* (*5*). We detected no antibodies to the *M. bovis* antigens MPB83 or ESAT6/CFP10 complex in serum samples tested with the Dual Path Platform VetTB assay (Chembio Diagnostic Systems, Inc., Medford, NY, USA) (*6*). PCR analyses confirmed *M. bovis* infection, both directly from the lung tissue and indirectly from mycobacteria culturing of lung tissue samples, as previously described (*7*,*8*).

Because black rhinoceros have been shown to be susceptible to TB, it is not unexpected to diagnose bovine TB in a free-ranging rhinoceros in an area with a high prevalence of TB in other wildlife species (*4*). Risk factors such as environmental load of mycobacteria, presence of concurrent disease, and other stressors (including malnutrition associated with drought) might result in progression of *M. bovis* infection. Although the source of infection for the animal we describe is unknown, no known exposure to humans or livestock has occurred. It is possible that interaction with other infected wildlife, including African buffalo, which are considered maintenance hosts of bovine TB, or environmental contamination at shared water holes and feeding sites might have resulted in pathogen contact (*8*,*9*).

Occurrence of *M. bovis* infection in a free-ranging black rhinoceros in KNP might have substantial consequences for conservation programs. The risk for disease transmission between isolated, small populations of critically endangered species could hinder future translocation of these animals. Further risk assessments are needed to investigate the importance of this finding.

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Zika Virus Vector Competency of Mosquitoes, Gulf Coast, United States

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Zika virus has recently spread throughout the Americas. Although *Aedes aegypti* mosquitoes are considered the primary vector, *Culex quinquefasciatus* and mosquitoes of other species may also be vectors. We tested *Cx. quinquefasciatus* and *Ae. taeniorhynchus* mosquitoes from the US Gulf Coast; both were refractory to infection and incapable of transmission.

Although most human Zika virus infections produce no symptoms or only mild febrile illness, the association with microcephaly and other severe congenital defects has caused a public health crisis since the virus arrived in the Americas. Part of the concern is local, mosquitoborne transmission in the United States (*1*). *Aedes* (*Stegomyia*) *aegypti* mosquitoes are believed to be the primary urban Zika virus vectors, according to laboratory transmission studies (*2*) including vertical (*3*) and natural Zika virus infections detected in Malaysia (*4*) and during a 2015 Mexico outbreak (*5*). This highly anthropophilic mosquito occurs nearly throughout the tropics and subtropics, including the southern United States. However, in many tropical and subtropical regions, the most abundant urban mosquito is *Culex quinquefasciatus*. One experimental study found that *Cx. quinquefasciatus* mosquitoes from China are capable of Zika virus transmission (*6*), and others found that mosquitoes of this species and the closely related *Cx. pipiens* are refractory to Zika virus infection (*2*). Surveillance during an outbreak in Mexico also found no evidence of natural *Cx. quinquefasciatus* mosquito infection in regions of active transmission (*5*).

One US region at highest risk for Zika virus circulation is the Gulf of Mexico coast (Gulf Coast), especially Houston, Texas, which is a major hub for air transportation and has large populations of *Ae. aegypti* mosquitoes. Evidence of past dengue virus circulation (*7*) also suggests permissive conditions for Zika virus transmission. However, the most abundant mosquitoes immediately along the Gulf Coast are typically salt marsh species, such as *Ae.* (*Ochlerotatus*) *taeniorhynchus*, a competent vector for arboviruses, including Venezuelan equine encephalitis virus. Mosquitoes of this species are widely distributed in North, Central, and South America, and their mammalophilic feeding behavior could enable transmission of arboviruses among humans (*8*).

To determine if *Cx. quinquefasciatus* mosquitoes are capable of Zika virus transmission, we fed cohorts of 50 mosquitoes (colonized and reared in an insectary) artificial blood meals containing 10^4 to 10^6 focus-forming units (FFU)/mL of virus prepared in Vero cell cultures. Fully engorged mosquitoes were incubated at 27°C and 80% humidity and provided aqueous sucrose ad libitum. Multiple Zika virus strains were fed to the mosquito cohorts: FSS13025 (2010 Cambodia, closely related to strains from the Americas), DAKAR41525 (1985 Senegal), and MEX1–7 (isolated from a 2015 outbreak in Mexico) (*5*). On days 3, 7, and 14 after mosquito feeding, we homogenized bodies and legs from 20 mosquitoes and tested them for Zika virus by focus-forming assay; on days 7 and 14, we also tested saliva.

Because natural blood meals from viremic animals are typically more infectious for mosquitoes than are artificial meals (*9*), we allowed 3 groups of *Cx. quinquefasciatus* mosquitoes to feed on FSS13025-infected type I interferon-receptor knockout A129 mice on postinfection days 1, 2, and 3, corresponding to viremia titers of 10^4, 10^7, and 10^6 FFU/mL, respectively, as determined by back-titration of mouse blood collected immediately after feeding. A separate mouse was used for each infection. On days 3, 7, and 14, we subjected mosquito bodies, legs, and saliva to focus-forming assay. All samples were also negative for Zika virus (Table).

To preclude the possibility that laboratory colonization diminished *Cx. quinquefasciatus* mosquito competence for Zika virus transmission, we collected F2 mosquitos from the Houston area and also allowed them to feed on A129 mice 2 days after infection with FSS13025, MEX1–7, or Puerto Rico strain PRVABC59, with viremia titers of 10^7, 10^6, and 10^7 FFU/mL, respectively. None of the bodies, legs, and saliva samples collected 14 days after feeding were positive for Zika virus.

Ae. taeniorhynchus mosquitoes were also tested for Zika virus transmission competence. Colonized mosquitoes were fed artificial blood meals containing 10^6 FFU/mL Zika virus (strain MEX1–44), and on days 10 (n = 20) and 17 (n = 20), salivary glands, legs, and midguts were dissected and screened for virus by infectious assays (*3*). None of the mosquito samples was positive for Zika virus (Table).

Our results concur with those of others showing the inability of Zika virus to infect *Culex* spp. mosquitoes (*2*).

[1]These authors contributed equally to this article.

Table. Potential mosquito vectors of southern United States that showed no infection, dissemination, or transmission of Zika virus*

Virus strain	Mosquito species/strain	Blood meal	Dose, log_{10} FFU/mL	No./time point	Days tested after feeding
MEX 1–44 (Mexico 2015)	*Culex quinquefasciatus* (colonized)	Artificial	6	20	10, 17
	Aedes taeniorhynchus (colonized)	Artificial	6	20	10, 17
DAK AR 41525 (Senegal 1985)	*Cx. quinquefasciatus* (colonized)	Artificial	4, 5, 6	20	3, 7, 14
FSS 13025 (Cambodia 2010)	*Cx. quinquefasciatus* (colonized)	Artificial	4, 5, 6	20	3, 7, 14
	Cx. quinquefasciatus (Houston F2)	Murine	4, 6, 7	5	3, 7, 14
MEX 1–7 (Mexico 2015)	*Cx. quinquefasciatus* (colonized)	Artificial	4, 5, 6	20	3, 7, 14
	Cx. quinquefasciatus (Houston F2)	Murine	6	26	14
PRABC59 (Puerto Rico 2015)	*Cx. quinquefasciatus* (Houston F2)	Murine	7	21	14

*Infection, dissemination, and transmission rates were all 0. FFU, focus-forming units.

We also found that *Ae. taeniorhynchus* mosquitoes from the Gulf Coast are refractory to Zika virus infection. The Zika virus strains and actual stocks used for our experiments were infectious for *Ae. aegypti* mosquitoes in other experiments (C. Roundy et al., unpub. data), indicating that our negative findings for *Cx. quinquefasciatus* and *Ae. taeniorhynchus* mosquitoes represent truly refractory phenotypes. These results, along with findings from an outbreak in southern Mexico (*5*), support the conclusion that *Ae. aegypti* mosquitoes are the primary urban Zika virus vectors. However, regional variation in competence could be reflected in the study from China that shows Zika virus presence in saliva after experimental infection (*6*). Additional research is needed to understand whether this putative geographic variation reflects mosquito genetics or other intrinsic factors, such as microbiome or microvirome populations within this species. Because some studies indicate that *Cx. quinquefasciatus* mosquitoes are more ornithophilic than mammalophilic, including in parts of China (*10*), their feeding habits in regions where they are transmission competent require evaluation to assess their true capacity as vectors.

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Mr. Hart and Mr. Roundy are graduate students in the Human Pathophysiology and Translational Medicine program at the University of Texas Medical Branch. Their research interests include vector biology and arboviruses transmitted by mosquitoes.

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Correction: Vol. 22, No. 7

The name of author Felix Drexler was misspelled in Hepatitis E Virus Infection in Dromedaries, North and East Africa, United Arab Emirates, and Pakistan, 1983–2015 (A. Rasche et al.). The article has been corrected online (https://wwwnc.cdc.gov/eid/article/22/7/16-0168_article).

Correction: Vol. 23, No. 2

The key in the Figure 1 inset should have referred to hepatitis A and E in Changing Epidemiology of Hepatitis A and Hepatitis E Viruses in China, 1990–2014 (X. Ren et al.). The article has been corrected online (http://wwwnc.cdc.gov/eid/article/23/2/16-1095_article).

Edvard Munch (1863–1944) Das Kind und der Tod, 1899 (The Child and Death, 1899) (detail). Oil on canvas. 39.4 in by 35.4 in/100 cm × 90 cm. Kunsthalle Bremen, Der Kunstverein, Bremen, Germany

Keeping It in the Family: the Childhood Burden of Tuberculosis

Terence Chorba, John Jereb

> "My art must be seen against the background of the heavy freight of my inheritance,–tuberculosis on Mother's side, mental illness on Father's side (Grandfather's phthisis),–my art is a self confession…" "The illness followed me all through my childhood and youth,–the germ of consumption placed its blood-red banner victoriously on the white handkerchief."
>
> —Edvard Munch

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Edvard Munch, born in December 1863, was the second of 5 children of Laura Bjølstad and Christian Munch, a physician, in Løten, Norway. As an infant, he moved with his family to Christiania (now Oslo). There Laura died in 1868 of tuberculosis (TB), after which Christian dealt with profound depression. At the time of Laura's death, 14 years before Robert Koch announced that *Mycobacterium tuberculosis* was the cause of the disease, an estimated 285 persons per 100,000 died of phthisis (pulmonary TB or a similar progressive systemic disease) annually in Norway; most deaths occurred among those of child-bearing age. In 1896–1900, after the technique for diagnostic sputum smears was widely known and practiced, the death rate from TB in Norway was 415 per 100,000 for women 20–39 years of age; the difference from the earlier number perhaps reflected increased diagnostic acumen.

In his memoirs, Munch recalled the Christmas when, at age 5, he stood with his 6-year-old sister (Johanne Sophie) and his younger siblings at their mother's bedside. Sophie sang "Silent Night," and Laura kissed each child. Shortly thereafter, Laura died. Munch later portrayed the desperation of a child clutching her head at her mother's death, in *The Child and Death*, featured on this month's cover.

In *The Child and Death,* Munch captures the innocence of childhood disrupted by terrible circumstances, made more heartbreaking because the mother's death portends the daughter's death—the infection has been transmitted already. Munch knew what awaited his sister: her wide-eyed gaze shames the viewer/voyeur who has drawn closer to inquire about warm flesh tones against a background of gray-blue pallor, while bloody carmine smudges the bed and creeps around the girl.

The World Health Organization (WHO) has estimated that 9.7 million children (aged <15 years) are now orphans because of TB. In addition to the social and psychological burden of TB, children themselves account for a considerable portion of the associated morbidity and mortality. WHO estimates that 10.4 million new (incident) TB cases occurred in 2015, of which 5.9 million were in men, 3.5 million in women, and 1.0 million in children. The diagnosis and treatment of childhood TB are often problematic. Adequate sputum samples are difficult to obtain from children, thus hindering timely diagnosis. There is also relative lack of drug formulations for children, despite recent introductions of user-friendly fixed-dose combinations.

Sophie died from TB at age 15, a year after Munch himself took ill with the disease. Munch recalled the pathos of Sophie's death in the painting *The Sick Child* (1886), featured as EID's cover art in March 2011. Munch's account of his own illness is poignant:

> "'Papa the stuff I am spitting is so dark.'
>
> 'Is it, my boy?'
>
> He brought the candle….Next time I spat on the sheet to see what it was.
>
> 'It is blood Papa.'
>
> He stroked my hair – 'Don't be afraid, my boy.'
>
> So I had tuberculosis. There was so much talk about it. When you spat blood you had tuberculosis….
> 'Don't be frightened boy,' Father said again.
>
> 'When you spit blood you have tuberculosis,' I said and I coughed again and got more blood."

Munch's survival was unexpected: in the pre-antimicrobial drug era, the case-fatality rate for TB was 70%. Although Munch also nearly died of influenza in the pandemic of 1918–19, he survived, recovered, and died in 1944, at age 80, at his country home in Ekley, Norway.

Globally the epidemics of drug-resistant TB, multidrug-resistant TB, and extensively drug-resistant TB are formidable. Almost 10% of *M. tuberculosis* isolates in the United States and 20% of isolates worldwide are resistant to at least one first-line TB drug, mostly to isoniazid. Drug resistance is associated with greater morbidity, accounts for almost 25% of global TB mortality, and requires treatment that is more costly, more difficult, and of greater duration. These circumstances threaten to reverse the antimicrobial gains against TB, pushing us toward a world that may more resemble the pre-antibiotic era in which Edvard Munch's mother and sister died, and in which he somehow survived to bring us the ghosts of his memories.

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- Treatment Failure of Dihydroartemisinin-Piperaquine for *Plasmodium falciparum* Malaria, Vietnam

Complete list of articles in the April issue at
http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

March 29–31, 2017
SHEA
Society for Healthcare Epidiemiology of America
St Louis, MO, USA
http://www.shea-online.org/

April 10–12, 2017
World Vaccine Congress
Washington, DC, USA
http://www.terrapinn.com/conference/world-vaccine-congress-washington

April 22–27, 2017
ECCMID
European Congress of Clinical Microbiology and Infectious Diseases
Vienna, Austria
http://www.eccmid.org/

June 1–5, 2017
ASM
American Society for Microbiology
New Orleans, LA, USA
http://www.showsbee.com/fairs/25161-ASM-Microbe-2017.html

June 4–8, 2017
Council of State and Territorial Epidemiologists
2017 Annual Conference
Boise, ID, USA
http://www.csteconference.org/2017/

June 19–21 2017
Transmission of Respiratory Viruses
Harbour Grand Hong Kong
https://transmission2017.med.hku.hk/mass_email.html

March 1–4, 2018
18th International Congress on Infectious Diseases (ICID)
Buenos Aires, Argentina
http://www.isid.org/icid/

Announcements

To submit an announcement, send an email message to EID Editor (eideditor@cdc.gov). Include the date of the event, the location, the sponsoring organization(s), and a website that readers may visit or a telephone number or email address that readers may contact for more information.

Announcements may be posted on the journal Web page only, depending on the event date.

Earning CME Credit

To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions (with a minimum 75% passing score) and earn continuing medical education (CME) credit, please go to http://www.medscape.org/journal/eid. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.org. If you are not registered on Medscape.org, please click on the "Register" link on the right hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to http://www.ama-assn.org/ama/pub/about-ama/awards/ama-physicians-recognition-award.page. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for AMA PRA Category 1 Credits™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit may be acceptable as evidence of participation in CME activities. If you are not licensed in the US, please complete the questions online, print the certificate and present it to your national medical association for review.

Article Title

Epidemiology of *Mycobacterium bovis* Disease in Humans in England, Wales, and Northern Ireland, 2002–2014

CME Questions

1. You are advising a UK public health department about anticipated *Mycobacterium bovis* cases. According to the national cohort study by Davidson and colleagues, which of the following statements about demographic features of *M. bovis* cases in England, Wales, and Northern Ireland from 2002-2014 is most accurate?

A. Annual case numbers decreased from 2002 to 2014
B. Incidence was 0.05 per 100,000
C. Incidence rate declined across time
D. Median age of UK-born cases increased across time

2. According to the national cohort study by Davidson and colleagues, which of the following statements about the demographic and clinical characteristics of human tuberculosis caused by *M. bovis* compared with those caused by *M. tuberculosis* is correct?

A. *M. tuberculosis* notified persons were more likely to be 65 years and older
B. *M. tuberculosis* notified persons were more likely to be of an ethnic group other than the Indian Subcontinent
C. *M. tuberculosis* notified persons were more likely to live in a rural area
D. The strongest risk factor for *M. bovis* was working in an agricultural or animal-related occupation (adjusted odds ratio, 29.5; 95% confidence interval, 16.9–51.6)

3. According to the national cohort study by Davidson and colleagues, which of the following scenarios regarding potential exposures, including genotyping comparison between human cases, that may indicate *M. bovis* acquisition in humans would most likely occur?

A. One-quarter of persons had a known risk exposure for *M. bovis* acquisition
B. Living in a rural area was the most frequently reported risk exposure
C. Half of persons reported contact with a human case of tuberculosis within 5 years of *M. bovis* diagnosis
D. From 24 loci mycobacterial interspersed repetitive unit (MIRU) variable-number tandem-repeat (VNTR) strain typing data available between 2010 and 2014, 48.7% of persons (46 UK-born; 9 non–UK-born) were in 15 *M. bovis* strain-type clusters

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

Earning CME Credit

To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions (with a minimum 75% passing score) and earn continuing medical education (CME) credit, please go to http://www.medscape.org/journal/eid. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.org. If you are not registered on Medscape.org, please click on the "Register" link on the right hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to http://www.ama-assn.org/ama/pub/about-ama/awards/ama-physicians-recognition-award.page. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for AMA PRA Category 1 Credits™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit may be acceptable as evidence of participation in CME activities. If you are not licensed in the US, please complete the questions online, print the certificate and present it to your national medical association for review.

Article Title

Three Cases of Neurologic Syndrome Caused by Donor-Derived Microsporidiosis

CME Questions

1. Your patient is a 53-year-old man who has recently undergone liver transplantation. According to the Centers for Disease Control and Prevention (CDC) investigation by Smith and colleagues, which of the following statements about the clinical presentation and course of 3 solid-organ recipients infected with *Encephalitozoon cuniculi* from a single infected donor is most accurate?

A. All recipients had gastrointestinal symptoms
B. There were no deaths
C. No patient had tremor or gait disturbance
D. The heart/kidney and contralateral kidney recipients were hospitalized with encephalitis

2. According to the CDC investigation by Smith and colleagues, which of the following statements about diagnostic testing and confirmation of *E. cuniculi* infection in 3 solid-organ transplant recipients is correct?

A. Tissue polymerase chain reaction (PCR) showed E. cuniculi in renal allograft tissue from only 1 kidney recipient
B. *E. cuniculi* could not be detected in the central nervous system of any recipient
C. Urine PCR result was positive for *E. cuniculi* in only 1 recipient
D. If clinical suspicion exists, clinical laboratories can accomplish rapid diagnosis by light microscopy of urine, stool, or other specimens using a modified trichrome stain

3. According to the CDC investigation by Smith and colleagues, which of the following statements about management and clinical implications of *E. cuniculi* infection in 3 solid-organ transplant recipients is correct?

A. Extensive collaboration is needed to identify donor-derived disease transmission and to facilitate appropriate treatment and management
B. The only pathogen that the CDC has previously implicated in encephalitis cases transmitted through solid-organ transplantation is the West Nile virus
C. Encephalitozoonidae are resistant to albendazole
D. Patients received only 2 weeks of antibiotic treatment

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

Visit www.cdc.gov/vaccines/parents. Learn about the vaccines your baby needs from a reliable source. The CDC's website explains the 14 diseases vaccines prevent, CDC's recommended schedule, possible side effects, how to comfort your baby during vaccine visits and more. Talk to your child's doctor, and visit our website to get the facts about vaccines.

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Centers for Disease Control and Prevention

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- Patient education materials
- Posters
- Fact sheets
- Flyers

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Preparación y respuesta para casos de emergencias
Agentes del terrorismo biológico, emergencias químicas, brotes, desastres naturales...

Salud ambiental
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Etapas de la vida y poblaciones
Adolescentes, bebés y niños, hombres, muheres, salud de las minorías...

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The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles, see below and visit http://wwwnc.cdc.gov/eid/pages/author-resource-center.htm.

Summary of Authors' Instructions

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Manuscript Preparation. For word processing, use MS Word. Set the document to show continuous line numbers. List the following information in this order: title page, article summary line, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, and figure legends. Appendix materials and figures should be in separate files.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Use terms as listed in the National Library of Medicine Medical Subject Headings index (www.ncbi.nlm.nih.gov/mesh).

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje.org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

Tables. Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of boldface. Tables should be no wider than 17 cm. Condense or divide larger tables. Extensive tables may be made available online only.

Figures. Submit editable figures as separate files (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .tif or .jpeg files. Do not embed figures in the manuscript file. Use Arial 10 pt. or 12 pt. font for lettering so that figures, symbols, lettering, and numbering can remain legible when reduced to print size. Place figure keys within the figure. Figure legends should be placed at the end of the manuscript file.

Videos. Submit as AVI, MOV, MPG, MPEG, or WMV. Videos should not exceed 5 minutes and should include an audio description and complete captioning. If audio is not available, provide a description of the action in the video as a separate Word file. Published or copyrighted material (e.g., music) is discouraged and must be accompanied by written release. If video is part of a manuscript, files must be uploaded with manuscript submission. When uploading, choose "Video" file. Include a brief video legend in the manuscript file.

Types of Articles

Perspectives. Articles should not exceed 3,500 words and 50 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.

Synopses. Articles should not exceed 3,500 words in the main body of the text or include more than 50 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (not to exceed 150 words), a 1-line summary of the conclusions, and a brief biographical sketch of first author or of both authors if only 2 authors. This section comprises case series papers and concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research. Articles should not exceed 3,500 words and 50 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should not exceed 3,500 words and 50 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and biographical sketch. Dispatches are updates on infectious disease trends and research that include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit. Include biographical sketch.

Research Letters Reporting Cases, Outbreaks, or Original Research. EID publishes letters that report cases, outbreaks, or original research as Research Letters. Authors should provide a short abstract (50-word maximum), references (not to exceed 10), and a short biographical sketch. These letters should not exceed 800 words in the main body of the text and may include either 1 figure or 1 table. Do not divide Research Letters into sections.

Letters Commenting on Articles. Letters commenting on articles should contain a maximum of 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references (not to exceed 15) but no abstract, figures, or tables. Include biographical sketch.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

Conference Summaries. Summaries of emerging infectious disease conference activities (500–1,000 words) are published online only. They should be submitted no later than 6 months after the conference and focus on content rather than process. Provide illustrations, references, and links to full reports of conference activities.

Online Reports. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

Photo Quiz. The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

Etymologia. Etymologia (100 words, 5 references). We welcome thoroughly researched derivations of emerging disease terms. Historical and other context could be included.

Announcements. We welcome brief announcements of timely events of interest to our readers. Announcements may be posted online only, depending on the event date. Email to eideditor@cdc.gov.

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